

Male Hormonal Contraception

The Effects of Progestogens on the Hypothalamic-Pituitary-Gonadal axis in the Human Male

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Declaration

I declare that the studies undertaken in this Thesis are the result of my own investigation with the exception of Chapter 3 in which data from a multi-centre study are presented of which I was responsible for the Edinburgh cohort. Serum hormone assays in Chapters 2 and 3 were undertaken by NV Organon as were etonogestrel assays in Chapter 4. Statistical analysis in Chapter 3 was performed by NV Organon.

This work has not been submitted for candidature for any other degree.

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(Candidate)

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Abstract of thesis

With a third of the World's population relying on male methods of contraception, there is a need to expand choice for couples relying on male methods. Combined testosterone and progestogen preparations suppress gonadotrophin secretion and spermatogenesis, and are a promising approach to male hormonal contraception. The Edinburgh cohort (20 subjects) of a multicentre study investigating the efficacy in suppression of spermatogenesis and gonadotrophins with 300µg oral etonogestrel (ENG) and intramuscular testosterone decanoate (TD) (400mg 4 or 6 weekly) over 48 weeks is reported. Despite persisting sub-physiological trough testosterone concentrations, profound spermatogenic and gonadotrophin suppression was observed and was greater in Group I receiving 400mg TD every 4 weeks than Group II (400mg TD every 6 weeks).

Depot gestogen preparations may permit 'dose-sparing' thus minimising adverse metabolic effects, and allowing a more convenient dose interval. This regime was further investigated using ENG implants and i.m. TD for 48 weeks in a multi-centre study (130 subjects). Subjects received 204mg ENG implants (equivalent to 3 Implanon®) and either 400mg TD every 4 weeks, 6 weeks or 600mg 6 weekly for a period of 48 weeks. A similar profound suppression of spermatogenesis and gonadotrophins was observed again, with a lesser suppression in the lower testosterone group receiving 400mg TD 6 weekly.

The effects of the same dose of etonogestrel implants was investigated in a further study (15 subjects) with a different testosterone preparation, 400mg pellets at 12 weekly intervals. Suppression of spermatogenesis was greater than the other regimes investigated with sperm concentrations of $<1 \times 10^6$ M/ml in all men by 16 weeks of treatment and eventual azoospermia in all subjects. Testosterone levels remained in

the physiological range throughout. In contrast to the other regimes, there were no adverse metabolic effects with no weight gain, change in body composition, or decline in HDL-C concentrations.

The underlying mechanisms of the antigonadotrophic effects of gestogens in the male were investigated. Gestogens have affinity for both androgen and progesterone receptors but the relative contribution of action at these two receptors in gonadotrophin suppression remains unclear. The effects of progesterone, with no significant androgen-receptor affinity were compared to desogestrel, with relatively low affinity for the androgen receptor, on gonadotrophin secretion in normal men. Twenty healthy men were randomly allocated to the two treatment groups receiving either 50mg progesterone i.m. or 300 μ g desogestrel p.o. daily for 7 days. Frequent blood sampling over 12 hrs was undertaken before and after drug administration. GnRH (100 μ g i.v.) was administered 2 hrs before the end of the frequent sampling period. Both progesterone and desogestrel administration resulted in decreases in the concentration of both LH and FSH secretion, as well as testosterone. Analysis of the pulsatile nature of LH secretion indicated that both treatments reduced LH pulse amplitude, and that progesterone reduced LH pulse frequency. Progesterone but not desogestrel treatment also reduced the increase in LH secretion in response to GnRH. The effects of progesterone were at least as marked as those of a maximally-effective dose of desogestrel. As progesterone has negligible affinity for the androgen receptor, these results suggest that the suppressive effects of synthetic gestogens on gonadotrophin secretion in the male are not due solely by nature of their androgenicity but are mediated via the progesterone receptor.

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CHAPTER 1

MALE REPRODUCTIVE AXIS AND HORMONAL CONTRACEPTION

Introduction

Advances in contraception have been almost exclusively female-directed despite the widespread use of male methods worldwide and increasing calls for the burden of contraception to be more evenly shared. With over 150 million couples worldwide relying on male methods (United Nations, 2000) there is clearly a need to expand the current limited choices of vasectomy or condoms. Of the several potential approaches to novel male methods, the hormonal approach is the nearest to fruition. The basis of this approach lies in the suppression of gonadotrophin secretion resulting in a fall in sperm production. A further consequence is the suppression of testicular steroidogenesis requiring the co-administration of androgen. It is interesting that the reversible suppression of spermatogenesis was demonstrated several decades ago (McCullagh and McGurl, 1939), yet the introduction of a new contraceptive method for men has not occurred. The use of testosterone as a reversible contraceptive agent in men has been demonstrated in studies undertaken by World Health Organisation over the last decade. However, an agent that results in universal azoospermia without significant side-effects remains elusive. Consequently, combination approaches with progestogens, anti-androgens, 5 α -reductase inhibitors and gonadotrophin releasing hormones (GnRH) antagonists have been evaluated with the aim of improving contraceptive efficacy. Different methods of androgen delivery are also being developed in order to minimize extra-testicular effects and improve acceptability. This chapter will overview the relevant areas of the male reproductive axis, the biological

actions of androgens, and then focus on efforts to date to develop a safe, acceptable, efficacious hormonal contraceptive for men.

1.1 THE TESTIS

In the majority of mammalian species, the testes are intra-scrotal following their embryological descent. This has functional importance in lowering temperature, which is necessary for normal spermatogenesis. Their vascular supply is attained proximally, close to the aortic origin of the renal arteries. Venous drainage commences as the pampiniform plexus of veins terminating at the left renal vein and inferior vena cava on the right. This vascular arrangement further facilitates the lowering of temperature, as arterial blood approaches the testis via a counter-current exchange mechanism.

Within the testis, the interstitium surrounds the seminiferous tubules and is divided by septae from the surrounding outer dense connective tissue covering, the tunica albuginea. In addition to vascular and lymphatic vessels that are essential for the movement of hormones and nutrients into and out of the testis, the steroidogenic cell of the testis responsible for androgen output, the Leydig cell, lie within this connective tissue. Within the seminiferous tubule, developing spermatogonia lie adjacent to the basement membrane interspersed with the supporting Sertoli cells of somatic origin.

Spermatogenesis is a complex process which describes the sequence of events whereby immature germ cells undergo division, differentiation and meiosis to give rise to elongated haploid spermatids. This takes place within the seminiferous tubule. This process is coordinated by the organisation of the testis, whereby adjacent Sertoli cells

are adjoined by intercellular tight junctions constituting the blood-testis barrier. A single Sertoli cell maintains morphological intimacy with various germ cells at different stages of development through arborizing branches of their cytoplasm. This allows extensive communication between Sertoli and germ cells at a biochemical and molecular level throughout spermatogenesis. The various generations of germ cells are arranged in strict cellular associations, constituting the cycle of the seminiferous epithelium. This gives rise to a series of defined species-specific stages which shall be considered later.

1.2. SPERMATOGENESIS

1.2.1 Testis determination

In humans and other mammals sex is determined at fertilisation, the Y bearing sperm giving rise to XY males. However, the pathway of development from the bipotential indifferent gonad of the embryo into the testis remains incompletely understood. This stage was originally thought to be controlled by the Tdy (testis determining gene) on the short arm of the Human Y-chromosome through the plasma membrane protein, H-Y histocompatibility antigen. The gene that triggers this cascade has now been identified as SRY (sex determining region, Y) (Sinclair *et al.*, 1990), mutations in which are associated with failure in testicular determination in a condition known as 46, XY gonadal dysgenesis. However, accumulating evidence now suggests that sex determination cannot be explained by a simple switch, and the normal function of a number of non-Y-chromosomal genes 'downstream' to this switch is required including SOX9, WT1, SF-1 and DAX-1 (reviewed in (Veitia *et al.*, 2001)).

1.2.2 The Sertoli Cell

In the human embryo, by 5 to 6 weeks post-fertilization, primordial germ cells have migrated from the endoderm of the yolk sac to the genital ridge. The first morphological identifiable event in the development of the fetal testis is the development of Sertoli cell precursors. At 7 to 8 weeks, strands of germ cells and Sertoli cell precursors form testicular sex cords, which are the precursors of the seminiferous tubules (Waters and Trainer, 1996). This precedes human fetal Leydig cell development which begin cytodifferentiation from 8 weeks and peak at around 14-18 weeks gestation. During testicular differentiation, while the testis is becoming organised into cords, the Sertoli cell begins to secrete AMH (Anti-Mullerian Hormone) resulting in regression of the Mullerian duct leaving vestigial remnants. Its importance is evident from gene mutations encoding for AMH and the AMH receptor resulting in impairment of sexual differentiation (Belville *et al.*, 2004).

The number of Sertoli cells in the testis determines future fertility in the male, as each Sertoli cell can only support a finite number of germ cells (Russell *et al.*, 1990; Sharpe, 1994) and Sertoli cell number is directly related to daily sperm production (Johnson *et al.*, 1984). In the majority of species studied to date, the replication of Sertoli cells is limited to late fetal life and the neonatal period (Sharpe, 1994). Certainly in man, Sertoli cell number increases rapidly in the first 3-12 months of life (Cortes *et al.*, 1987) mirroring the elevation in circulating blood FSH levels. FSH has been shown to stimulate proliferation of rat fetal Sertoli cells in vitro (Orth, 1984, 1986; Sasaki *et al.*, 2000) although this has still to be demonstrated in the human. Similarly in the neonatal period, FSH increases the rate at which Sertoli cells divide in rats: inhibition of FSH

production by GnRH hormone antagonists in the neonatal period permanently reduces Sertoli cell number and daily sperm production (van den Dungen *et al.*, 1990; Sharpe *et al.*, 1998). Therefore both these periods are of great importance in determining the future fertility of the male.

In addition to regulating the process of spermatogenesis, the Sertoli cell provides both physical and biochemical support for the surrounding germ cells and is critically important for the transport of substances into the seminiferous tubule. The presence of inter-Sertoli cell junctions which develop during puberty, divides the seminiferous epithelium into 2 compartments; a basal one containing spermatogonia and preleptotene spermatocytes, and an adluminal one containing the subsequent stages of spermatocyte development. These junctions represent the site of the blood-testis barrier and by preventing intercellular transport they represent a highly selective permeability barrier specific to the Sertoli cell. Several molecules and signalling pathways including transforming growth factor β 3, occludin and Protein Kinases A and C appear to be involved with the regulation of Sertoli cell junction dynamics. However, the exact physiological relationship between these intricate dynamics and the process of spermatogenesis needs to be further elucidated (reviewed in (Cheng and Mruk, 2002; Lui *et al.*, 2003)). Following sexual maturation, the Sertoli cell secretes seminiferous tubule fluid under the control of FSH (Jegou *et al.*, 1982; Jegou *et al.*, 1983). Androgen Binding Protein (ABP), which binds both testosterone and DHT with high affinity, is produced by the Sertoli cell of many species (French and Ritzen, 1973; Ritzen *et al.*, 1981). It may have a role of ensuring adequate stores of testosterone within the tubule thus stabilizing fluctuations in Leydig cell output. Further

circumventing the blood-testis barrier, the Sertoli cell produces a number of other plasma proteins including albumin, transferrin and plasminogen activator (Lacroix *et al.*, 1977; Wright *et al.*, 1981; Huggenvik *et al.*, 1984). However, the exact role of these substances in the testis remains unclear.

1.2.3 Germ cell development

Germ cell development involves a complex series of events, each cell type identified on the basis of differing morphology. Spermatogonia are exclusively located around the basement membrane of the tubule and give rise to two cell populations: one that proceeds through meiosis and the other that retains its stem cell function. In the human male there are 3 types of recognisable spermatogonia: types A dark, A pale and B, which are considered to be committed to differentiation. Thus proliferation of spermatogonia produces the source of millions of type B cells available for entry into meiosis and the production of millions of sperm every day.

In response to an unknown signal, the type B spermatogonia lose their contact with the basement membrane becoming preleptotene primary spermatocytes. Their DNA is replicated thus beginning the two divisions of meiosis. During the prophase of the first meiotic division germ cells undergo a series of characteristic nuclear changes. Pairing of homologous chromosomes occurs during the zygotene phase, with completely paired chromosomes being characteristic in pachytene spermatocytes. Closely paired chromosomes begin to repel themselves during the diplotene phase and the germ cells then undergo the first meiotic division forming secondary spermatocytes. They quickly undergo a second meiotic division yielding round spermatids with haploid DNA and

chromosomal complement. Subsequently, the differentiation of the round spermatid to the mature elongated spermatid involves acrosome and flagellum development, chromatin condensation, nuclear protein change and elongation and removal of cytoplasm in the stage of spermiogenesis. The spermatid is then ready for release during spermiation.

1.2.4 Regulation of the seminiferous epithelium

Germ cell development is dependent on interaction between the Sertoli and germ cell and on the secretion of gonadotrophins by a functioning hypothalamic-pituitary axis. The action of LH on spermatogenesis is mediated indirectly through the production of androgen by the Leydig cell, which act via androgen receptors on Sertoli, Leydig and Peritubular cells (Anthony *et al.*, 1989; Bremner *et al.*, 1994). FSH acts through G-protein coupled surface receptors on the surface of Sertoli cells and influences the development of the immature testis by controlling Sertoli cell proliferation (Orth, 1993). The actions of both these pituitary hormones are not only essential for normal testicular development, but also for normal testicular function (Themmen and Huhtaniemi, 2000). Many proteins are secreted from the Sertoli cell in a stage-dependent manner demonstrating its ability to adapt to the changing needs of the germ cell (Parvinen, 1982). It has been generally thought that the Sertoli cell controls the germ cell cycle through this high level of cellular interaction. However recent evidence exploiting the technique of germ cell transplantation suggests that germ cell type dictates Sertoli cell function (Franca *et al.*, 1998).

It is well established that gonadotrophins are the major regulators of spermatogenesis and that androgens are essential for spermatogenesis (Roberts and Zirkin, 1991). First, let us consider LH which acts on the Leydig cell stimulating androgen production. Androgens thus influence the process of spermatogenesis through their interaction with their receptor on the seminiferous epithelium. In the hypogonadal *hpg* mouse, congenitally deficient in GnRH and consequently both LH and FSH, androgens alone have been shown to stimulate all phases of germ cell development (Singh *et al.*, 1995). Androgen receptors (AR) are located on the Sertoli, peritubular myoid and Leydig cells but not on the germ cell (Anthony *et al.*, 1989; Bremner *et al.*, 1994). The action of testosterone on somatic rather than germ cells has been recently demonstrated in germ cell transplantation models (Johnston *et al.*, 2001). On injecting spermatogonial cells from testicular feminized mice expressing no functional AR into seminiferous tubules of azoospermic mice expressing AR, quantitatively normal spermatogenesis was restored in recipient testis. Therefore, one can assume from this that the effects of testosterone and its derivatives on germ cells are mediated via communication from testicular somatic cells and murine spermatogenesis is not dependent on the direct action of androgen on germ cells. In humans many mutations in the AR have been documented (McPhaul, 1999) resulting in complete or partial androgen insensitivity syndrome which in its mildest form is associated with a phenotypically normal male with infertility (Wang *et al.*, 1998c). This evidence supports the need for normal level of androgen action for normal spermatogenesis. Intra-testicular testosterone concentrations are at a level that would exceed that to saturate the androgen receptor and would be markedly supraphysiological in the peripheral circulation. A gross impairment of spermatogenesis is observed in rats with the experimental reduction of

androgen levels to 20% of control levels (Awoniyi *et al.*, 1989; O'Donnell *et al.*, 1994). This is also seen in primates and humans following gonadotrophin withdrawal (O'Donnell *et al.*, 2001; McLachlan *et al.*, 2002b). The underlying reason is unclear but testis specific interactions between testosterone, the AR and co-factors are likely to exist.

Significant controversy remains regarding the role of FSH in the regulation of spermatogenesis. First let us consider evidence from animal studies suggesting that spermatogenesis can be completed in the absence of FSH. The FSH β subunit knock-out mouse results in normal sexual maturity and fertility however sperm output is only 40% of normal (Kumar *et al.*, 1997). Reduction in testis size may reflect an overall reduction in Sertoli cell number. However recent quantitative data suggest a compromised ability of the Sertoli cell to nurture germ cells with fewer round spermatids per Sertoli cell in comparison to the wild type (Wreford *et al.*, 2001). The targeted disruption of the FSH receptor (Dierich *et al.*, 1998) results in a similar phenotype. In addition, independent to Sertoli cell function there are additional defects in spermatogenesis with reduced nuclear condensation (Krishnamurthy *et al.*, 2000). Therefore, spermatogenesis continues in the absence of functional FSH action albeit at a reduced level. Similar evidence exists in humans with mutation of the FSH receptor in man resulting in variable reduction in sperm density but retention of fertility (Tapanainen *et al.*, 1997). However, unlike the knockout mouse, a case report of the FSH β ligand deficiency state resulted in the individual being azoospermic (Phillip *et al.*, 1998). This variation in phenotypes may result from differing mutations in the gene encoding the FSH β - subunit with resulting partial or complete deficiency of FSH.

There exists however considerable evidence to support an essential role for FSH in maintaining quantitatively normal spermatogenesis in man. In the hypogonadotrophic-induced male, hCG (LH) only restores spermatogenesis to 50% of baseline levels whereas both hCG and FSH treatment restores quantitatively normal spermatogenesis (Matsumoto *et al.*, 1986). Similarly in primates immunized against FSH, both sperm production and quality are impaired indicating a role for FSH in maintaining sperm function as well as number (Srinath *et al.*, 1983).

The bulk of evidence suggests that FSH regulates spermatogonial development (Zhengwei *et al.*, 1998) probably through the prevention of germ cell apoptosis (Sinha Hikim *et al.*, 1995) and also later spermiation (Saito *et al.*, 2000; McLachlan *et al.*, 2002b). It appears that in both primate models and in humans there is a striking inhibition of Type A to Type B spermatogonial development with maintenance of germ cell progression and a major loss in sperm output despite maintaining round spermatid number with the withdrawal of FSH. This suggests defective spermiation and consistent with this concept is the rapid fall in sperm count in less than 4 weeks evident in gonadotrophin withdrawn subjects under different male contraception regimes.

Although we have considered the effects of LH and FSH separately on the seminiferous epithelium, the division of these effects is complicated in that they share both structural and functional similarities and therefore the separation of these interconnected pathways is often confounded experimentally. Both LH and FSH share a common α -subunit and can be secreted simultaneously from the same pituitary gonadotrophs (Denef *et al.*,

1976) and pituitary FSH secretion is modulated by LH-mediated steroidal feedback (Kumar and Low, 1995). Furthermore, the testicular actions of both LH and FSH are targeted on the Sertoli cell, with the expression of FSH receptors (Rannikki *et al.*, 1995) and androgen receptors (Sar *et al.*, 1990) stimulated indirectly through the action of LH on Leydig cells. In recent transgenic mouse models, the action of LH and FSH have been selectively isolated to further clarify their roles in testicular development and function. In LH deficient *hpg* mouse models, it has been demonstrated that FSH alone can stimulate complete Sertoli cell proliferation independent of LH. However, FSH alone could not complete spermatogenic development and LH was critical in determining the full complement of germ cells and completing spermiogenesis (Allan *et al.*, 2004). The use of such models will no doubt in the future lead to further clarification of the complementary roles of LH and FSH in the complex process of spermatogenesis.

1.3. TESTICULAR STEROIDOGENESIS

1.3.1 The Leydig cell

The Leydig cell is responsible for the production of testosterone, the principal circulating androgen in man. It is postulated that there are two generations of Leydig cells, which are derived from the mesenchyme of the gonadal ridge. The first of these, the fetal generation, differentiate and begin to develop steroidogenic potential from around the 8th week of fetal life (Tapanainen *et al.*, 1981) and are responsible for masculinisation of the external genitalia. This occurs in the absence of gonadotrophin secretion and has close temporal relation to the secretion of maternal hCG (Clements *et*

al., 1976). This suggests that the secretion of chorionic gonadotrophin into the fetal serum may be the major stimulus to the fetal Leydig cell. After the 18th week, Leydig cells begin to involute and disappear entirely until a few weeks after birth (Huhtaniemi and Pelliniemi, 1992). The second generation, the adult Leydig cells, develop from connective tissue precursors concomitant with the pubertal secretion of gonadotrophins, and occupy about 5% of the total testicular volume within the interstitial space. However, this view of a biphasic pattern of development is not in keeping with biochemical studies demonstrating a triphasic pattern of testosterone production with peaks at 14-18 weeks of fetal life, 2-3 months of neonatal life and from puberty through adult life. Until recently there were relatively few data on this period of neonatal development. However, morphological evidence exists to support this period of development which seems dependent on the re-activation of the hypothalamic-pituitary axis (Prince *et al.*, 1998) and is concomitant with the neonatal rise in testosterone (Lunn *et al.*, 1994).

The Leydig cell has the ability to synthesise cholesterol from acetate, cholesterol acting as a substrate for steroidogenesis and the secretion of testosterone. In addition, it also secretes estradiol (20-30% of the total circulating estradiol in the human male) (Baird *et al.*, 1973). The enzymes involved are organized in the subcellular compartment, with the conversion of cholesterol to pregnenolone occurring in the mitochondria (Toren *et al.*, 1964; van der Vusse *et al.*, 1973) and the remainder in the smooth endoplasmic reticulum. Testosterone is then secreted into the intertubular space and absorbed by the blood vessels, lymphatics and seminiferous tubule. Before discussing the regulation of

Leydig cell steroidogenesis, the enzymatic pathways in the synthesis of testosterone from cholesterol shall be considered.

1.3.2 Steroidogenesis

Testosterone is the major circulating androgen in the male; the testes secrete 95% of the total daily production (6-7mg/day (Horton, 1978; Anderson *et al.*, 1996)) at the site of the Leydig cell. The precursor in steroid biosynthesis is cholesterol (C₂₇) which may be synthesised *de novo* in the Leydig cell or taken up from plasma lipoproteins, namely from the LDL fraction. The Leydig cell has a large capacity for endogenous cholesterol synthesis and has a limited capacity for uptake and storage. The transport of cholesterol from the plasma pool to the mitochondrial membrane is regulated by Steroidogenic Acute Regulatory Protein (StAR) (reviewed in (Stocco, 2000b, a)) and may be influenced by LH (Devoto *et al.*, 2002). Subsequently, the side-chain cleavage of cholesterol within the mitochondria by the cytochrome P₄₅₀ system is the primary and rate-limiting step (Waterman and Simpson, 1989) in the steroidogenic cascade leading to the formation of pregnenolone. The step-wise degradation of pregnenolone by the oxidative enzymes of the Cyt P₄₅₀ system within the endoplasmic reticulum converts this biologically inactive precursor into biologically active C₁₉ steroids. In contrast to rats in which the Δ 4 pathway is the more important (Samuels *et al.*, 1975), in humans the Δ 5 via 17 α -hydroxypregnenolone, dihydroepiandrosterone and 5-androstene-3 β ,17 β -diol is more important (Yanaihara and Troen, 1972; Weusten *et al.*, 1987). In normal conditions in the human male, this system is insufficient to convert all of the pregnenolone to testosterone resulting in the leakage of many progesterone intermediary metabolites.

1.3.3 Regulation of testicular steroidogenesis

Leydig cell function is regulated by both endocrine factors, namely pituitary gonadotrophins, and local paracrine/autocrine factors (reviewed in (Saez, 1994; Gnessi *et al.*, 1997)). LH/hCG is the main hormone, under physiological conditions, which regulates the Leydig cell and testosterone secretion in the testis. Following hypophysectomy, testicular regression occurs, an effect that is counteracted by administration of LH (Wing *et al.*, 1984). Furthermore, LH stimulation of testosterone secretion has been demonstrated both in vivo in the intact testis (Eik-Nes, 1970) and in vitro in Leydig cell cultures (Janszen *et al.*, 1976).

From the mid-trimester androgen secretion occurs in response to the interaction of LH with its receptor resulting in an increase in androgen secretion. LH acts via a classical protein hormone receptor mechanism interacting with a G-protein associated transmembrane receptor (Loosfelt *et al.*, 1989). Many inactivating deletions and mutations of the LH receptor have been described, resulting in a varying clinical phenotype, ranging from Leydig cell hypoplasia to pseudohermaphroditism (Huhtaniemi, 2000), dependent on the degree of inhibition of LH binding. In cases of gonadotrophin-independent sexual precocity in the male, activating mutations in the human LH receptor have been described, the cascade of testicular production from testicular Leydig cells being activated independent of LH secretion (Shenker *et al.*, 1993; Themmen *et al.*, 1998).

Although the LH receptor is coupled to both the adenylate cyclase and phospholipase C pathways, under physiological conditions most of the cellular effects of LH are

mediated through cAMP (Saez, 1994). Following ligand-binding, adenylate cyclase is activated (Abramowitz *et al.*, 1979) resulting in an increase in intracellular cAMP, activating protein kinase-mediated protein phosphorylation and the cellular effects of LH. Exposure of the Leydig cells to LH has a dual response: firstly, an acute steroidogenic effect with cAMP and steroid production within minutes and secondly, a long-term trophic effect. Several protein candidates have been postulated to be involved in the initial translocation of cholesterol from the cytosol to the inner mitochondrial membrane including sterol carrier protein (SCP2), steroidogenesis activating peptide (SAP) and peripheral benzodiazepine receptor (PBR). However more recently, it has been demonstrated that LH stimulates the synthesis of StAR (steroidogenic acute regulatory protein) in the Leydig cell in a time and dose-dependent manner, which plays a key role in hormonally induced steroid hormone biosynthesis. StAR stimulates the transfer of cholesterol from the outer to the inner mitochondrial membrane (reviewed in (Stocco, 2000b)), and is therefore instrumental in the first step of the biosynthesis of testosterone, the conversion of cholesterol to pregnenolone. Thereafter, steroid hormone biosynthesis takes place in the smooth endoplasmic reticulum.

The second type of response of LH is its long-term trophic effect on Leydig cell structure and function. In addition, LH can induce an early trophic response in Leydig cells (Hodgson and de Kretser, 1984), in particular the expression of proto-oncogenes from the *fos* and *jun* families, the role of which is unclear (Hall *et al.*, 1991). Evidence from both in vitro and in vivo studies (reviewed in (Saez, 1994)) has demonstrated that at low physiological doses, LH has a positive effect on the expression of genes encoding

for several Leydig cell functions. However, at high doses the trophic effects are preceded by a desensitisation period. LH down-regulates its own receptors (Catt and Dufau, 1973) by several mechanisms. These include internalisation-degradation of the receptor-ligand complex (Ghinea *et al.*, 1992), increased degradation of LH receptor mRNA (Chuzel *et al.*, 1995) and by inhibition of LH receptor gene transcription (Wang *et al.*, 1991; Chuzel *et al.*, 1995). The refractoriness of both cAMP and testosterone responses to LH has been demonstrated in *in vitro* studies, treating cultured pig Leydig cells with LH (Lejeune *et al.*, 1998). Interestingly, the cAMP response remains low for 24 hours and testosterone slowly recovers after reaching a nadir at 6 hours. Similarly, following the administration of a single injection of hCG in men, a biphasic secretory profile of testosterone secretion is observed with initial stimulation followed by inhibition and a subsequent peak at 48-72 hours (Padron *et al.*, 1980). In addition to increasing the mRNA, activity and function of StAR, LH is also involved in the regulation of the other enzymes in the steroidogenic cascade (O'Shaughnessy, 1991; Clark *et al.*, 1996; Lejeune *et al.*, 1998).

Although the majority of this discussion has focused on the role of LH, accumulating evidence now suggests that there may be other endocrine factors involved in the regulation of Leydig cell steroidogenesis including FSH (Gromoll *et al.*, 1996; Levalle *et al.*, 1998; Phillip *et al.*, 1998), and possibly glucocorticoids and prolactin (reviewed in (Saez and Lejeune, 1996)). Many potential regulatory molecules, produced locally within the testis have also been postulated to have a local regulatory role on Leydig cell function, with substantial evidence supporting insulin-like growth factor (IGF-1), platelet-derived growth factor (PDGF) and transforming growth factor (TGF β) as

potential paracrine/autocrine factors regulating Leydig cell function (reviewed in (Habert *et al.*, 2001)). The importance of such agents in the regulation of testosterone secretion remains unclear.

1.4 THE ANDROGEN RECEPTOR

The effects of androgens in development and in the adult are mediated via the androgen receptor protein (AR), encoded on the human X chromosome at position q11-q12. The gene encoding this receptor has been cloned (Lubahn *et al.*, 1988) and analysis of cDNA's have demonstrated that it is a member of the nuclear receptor family of transcription factors (Mangelsdorf *et al.*, 1995), which share both functional and structural homology. In common with other members of the nuclear receptor superfamily, it can be divided into 4 functional domains. It has a ligand-binding domain (LBD) to which testosterone and DHT bind, a DNA binding domain for recognising target DNA sequences, an N terminal transactivation domain and a hinge region. The binding of ligand to the AR leads to configurational change and transcriptional activation/inhibition thus transmitting extracellular into intracellular signals, by targeting promoter response elements and recruiting co-factors. It is now clear that its transcriptional activity is modulated by co-regulatory proteins (reviewed in (Heinlein and Chang, 2002)).

There has been considerable recent interest in genetic polymorphism, with our ever increasing knowledge of the human genome. The androgen receptor exhibits

polymorphism in exon 1, characterised by different numbers of CAG triplet repeats, resulting in variable lengths of a polyglutamine stretch. The number of CAG repeats in healthy populations ranges from 11-31; expansions to greater than 40 have been linked with neuro-degenerative disease in association with partial androgen insensitivity (La Spada *et al.*, 1991; Belsham *et al.*, 1992). It has been proposed that this polymorphism in the number of CAG repeats results in a subtle modulation of the transcriptional activity induced by the AR. Shorter CAG repeats impose a higher transcriptional activity and have an increased binding affinity for androgens (Feldman, 1997). Expanded CAG repeats lead to reduced transactivation (Tut *et al.*, 1997) and therefore an inverse relationship may exist between CAG repeats at the N-terminus of the AR and AR activity. Further studies have suggested that shorter CAG repeats are associated with an increased risk of prostate cancer (Nelson and Witte, 2002) and that an inverse relationship may exist between the number of repeats and sperm concentration (Tut *et al.*, 1997; von Eckardstein *et al.*, 2001) with longer residues being associated with infertility (Tut *et al.*, 1997). Thus the polymorphic nature of this exon of the AR may allow for subtle variation in androgenicity between individuals, modulating the action of androgens and therefore contributing to their widespread effects.

1.5 PERIPHERAL ANDROGEN ACTION

Androgens play an essential role in male reproductive function, developmentally with Wolffian duct and pubertal sexual development and in the maintenance of spermatogenesis. However, their actions are not restricted to this and they have a key role in a wide range of biological processes (Mooradian *et al.*, 1987). We have also discussed that testosterone is the principal circulating androgen and that it is secreted by testicular Leydig cells, mainly under the influence of LH stimulation and that down-regulation and desensitisation rapidly follows continuous stimulation. In this section, we will now consider the peripheral effects of androgens in specific tissues relevant to this thesis.

Testicular levels of testosterone are much higher (50 to 100 fold) than in peripheral plasma (Morse *et al.*, 1973; Sharpe, 1994). Daily production rates of 6-7mg/day have been derived from measuring the metabolic clearance rates of radiolabelled testosterone (Horton, 1978; Anderson *et al.*, 1996). However in another study using stable isotope dilution, lower daily production rates were estimated at 3.7 ± 2.2 mg/day (Vierhapper *et al.*, 1997). In addition to testosterone, a small proportion (20 - 25%) of estrogen and dihydrotestosterone (DHT) are directly secreted by the testis, the remainder being produced by peripheral tissues. Following secretion into spermatic venous blood, the majority is bound to the hepatic secreted sex hormone binding globulin (SHBG) (Dunn *et al.*, 1981) (which has a high affinity for both testosterone and DHT), and albumin (Pardridge, 1986). Only 1-3% of testosterone remains unbound.

1.5.1 Circadian rhythm of testosterone

The circadian rhythm of steroid hormones has been described for many years, the most well known being that of cortisol (Montanini *et al.*, 1988). The circadian variation in circulating testosterone has been well characterised in men (Piro *et al.*, 1973; Leymarie *et al.*, 1974; Rowe *et al.*, 1974; Guignard *et al.*, 1980; Bremner *et al.*, 1983; Tenover *et al.*, 1988; Gupta *et al.*, 2000). These studies report peaks of between 26-28 nmol/L at about 0600-0800hrs and nadir concentrations of approximately 17nmol/L at 1800-2000 hours. Since the metabolic clearance of testosterone, under basal conditions does not change (Tenover *et al.*, 1988), it is likely that the circadian variation is due to changes in testicular secretion. This has been demonstrated for both non-SHBG bound and total serum testosterone concentrations (Plymate *et al.*, 1989), and also that the circadian variation is blunted, if not absent, in the elderly. Of interest, significant correlation between the diurnal variation of testosterone and inhibin B has been demonstrated in healthy men (Carlsen *et al.*, 1999) with concentrations approximately 30-40% lower in the late afternoon in comparison to the morning. The underlying mechanisms of the generation of this rhythm are unknown. The pineal gland secreting melatonin and thus influencing the secretion of gonadotrophins and hypothalamic catecholamines such as norepinephrine (Moore, 1983) may play a role or there may be some additional paracrine/autocrine regulation at the level of the testis.

In view of the use of exogenous testosterone as an integral part of male hormonal contraceptive regimes, the effects of testosterone on peripheral tissues such as bone, prostate, lipid metabolism, sexual behaviour and body composition are of particular interest and will be considered in turn.

1.5.2 Effects on the prostate gland

The prostate gland is androgen dependent requiring testosterone for its growth, differentiation and function (Cunha *et al.*, 1987; Frick, 1998). Within the prostate, testosterone is converted to DHT by 5-alpha reductase and DHT is the major prostatic androgen directly modulating growth and gene expression within the gland. Data linking levels of endogenous testosterone and prostatic carcinoma are inconsistent (Carter *et al.*, 1995; Gann *et al.*, 1996; Gustafsson *et al.*, 1996) and there has been no established link with BPH (Gann *et al.*, 1995). However, because androgen withdrawal inhibits the progression of prostatic carcinoma (Santen, 1992), a link between androgens and this disease has been suspected. This gives rise to concern when using exogenous androgen therapy as a male contraceptive (Schally and Comaru-Schally, 1987; Pollard, 1990). However, controlled data is limited regarding the potential risks of exogenous androgen therapy in normal young men. There have been anecdotal reports of prostatic disease in older hypogonadal men on testosterone therapy (Jackson *et al.*, 1989) and a case of prostatic adenocarcinoma in a body-builder who abused anabolic steroids (Roberts and Essenhig, 1986). In hypogonadal men, testosterone treatment results in an increase in prostate volume comparable to age-matched controls (Behre *et al.*, 1994b). In a male contraceptive trial in patients receiving testosterone enanthate 200mg weekly for 12 months resulting in sustained rises in testosterone and DHT, a small rise in prostate volume was evident (Wallace *et al.*, 1993). However, the long-term effects of androgens on the prostate are unknown, emphasising the need for ongoing surveillance with further male hormonal contraceptive studies. This has been considered in the investigations that will be discussed in the studies of this thesis.

1.5.3 Effects on lipid metabolism

Men are more likely to develop coronary artery disease than women (Lerner and Kannel, 1986). Decreased HDL-C and increased LDL-C concentrations are recognised risk factors for coronary artery disease (Cunha *et al.*, 1987; Gordon and Rifkind, 1989; Jacobs *et al.*, 1990; Frick, 1998). In comparison to premenopausal females, men have lower high-density lipoprotein cholesterol (HDL-C) and higher triglyceride and low density lipoprotein cholesterol (LDL-C) concentrations. Estrogens stimulate HDL-C partly due to their suppressive effect on hepatic lipase activity (Jones *et al.*, 2002), the main enzyme responsible for HDL-C clearance, and this may attribute to the above gender differences.

Similarly, the decrease in HDL-C by androgens may be partly mediated by their stimulating effect on hepatic lipoprotein lipase (Krauss *et al.*, 1974; Glueck *et al.*, 1976). This decrease in HDL-C is observed following puberty (Kirkland *et al.*, 1987) in response to an increase in endogenous androgens, and with the administration of exogenous androgens and anabolic androgenic steroids (Solyom, 1979). Evidence from cross-sectional studies and interventional studies of sex hormones and lipoprotein levels has led to conflicting results, often reporting a positive correlation between HDL-C and testosterone levels (reviewed in (Wu and von Eckardstein, 2003)). However many of these studies may not reflect the direct regulatory effect of testosterone on lipid metabolism, but also the inverse relationship between testosterone levels and body fat and insulin resistance (Hergenc *et al.*, 1999) which would have a significant impact on HDL-C levels. Supraphysiological testosterone levels in men receiving testosterone enanthate (TE) as a male hormonal contraceptive prototype have consistently led to a

reduction in HDL-C levels (Bagatell *et al.*, 1994b; Anderson *et al.*, 1995; Meriggiola *et al.*, 1995). The role of endogenous androgen has been investigated by its suppression through GnRH antagonism, effectively inducing experimental hypogonadism. This results in an elevation of HDL-C which is not observed when a physiological dose of testosterone is also given (Goldberg *et al.*, 1985; Bagatell *et al.*, 1992; Behre *et al.*, 1994a), thus suggesting that this effect is mediated by endogenous androgen. The observation in boys during puberty with increasing testosterone levels that those with highest oestradiol levels had the greatest fall in HDL-C suggests that at least part of the androgen effect is mediated by oestrogens (Laskarzewski *et al.*, 1983). This is however not supported in studies in GnRH antagonised subjects receiving TE with or without the oral aromatase inhibitor testolactone (Bagatell *et al.*, 1994a). The group receiving testolactone, with markedly reduced estradiol levels had a significant reduction in HDL-C. Similar results are observed with the administration of TE with testolactone (Friedl *et al.*, 1990) also with a significant elevation in hepatic lipase activity. These studies indicate the importance of aromatised testosterone and the control of HDL-C by reciprocal alteration in hepatic lipase activity by gonadal steroids in healthy men.

1.5.4 Effects on sexual behaviour

The behavioural effects of the secretions from the testis have been documented for centuries. Leonardo da Vinci eloquently reported: "Testicles witness of coition. They contain in themselves ardour, that is they are the augmenters of the animosity and ferocity of the animals; and experience shows us this clearly in castrated animals, of which one sees the bull, the boar, the ram and the cock, very fierce animals, which after

being deprived of their testicles, remain very cowardly; so one sees a ram drive before it a herd of whethers, and a capon put to flight by a number of hens.”

It is now clear that testosterone is necessary although not sufficient alone to maintain normal libido. The critical level for sexual function has been suggested to lie around 10nmol/litre, however this exhibits significant inter-subject variation (Christiansen, 1998). There does seem to be some reproducibility linking declining testosterone levels with a decline in androgen-related sexual behaviour (Gooren, 1987). However, the relationship of testosterone to erectile function is more complex. Spontaneous erectile function such as nocturnal penile tumescence is androgen-dependent, declining in states of androgen deficiency and improving with androgen replacement (Davidson *et al.*, 1979; O'Carroll and Bancroft, 1984). Erectile response to visual erotic stimuli is however androgen-independent (Bancroft and Wu, 1983; Kwan *et al.*, 1983).

Other than isolated case-reports in castrated males, the role of testosterone on human sexual behaviour has been studied in hypogonadal men undergoing testosterone replacement, or in eugonadal men with supraphysiological testosterone levels after administration of pharmacological doses of testosterone. Indeed, the importance of androgens in sexual function is evident in studies in hypogonadal men with either 'pathological withdrawal', or undergoing exogenous androgen replacement (Davidson *et al.*, 1979; Skakkebaek *et al.*, 1981; Bancroft and Wu, 1983; Carani *et al.*, 1990; Snyder *et al.*, 2000). Administration of testosterone injections to eugonadal men with diminished libido results in a significant increase in sexual interest (O'Carroll and Bancroft, 1984). Supraphysiological testosterone levels in eugonadal men increase

sexual awareness and arousability, although this does not translate into modification in sexual behaviour (Anderson *et al.*, 1992; Bagatell *et al.*, 1994b). This has also been more recently demonstrated in a double-blind placebo controlled study administering 1000mg testosterone undecanoate to eugonadal men (O'Connor *et al.*, 2004). This study again demonstrated that elevation of testosterone to supraphysiological levels has no effect on sexual function. Therefore, physiological levels of testosterone do not appear to provide a maximal stimulus for sexual interest. Supraphysiological testosterone levels, although increasing arousability, do not result in any overt changes in sexual behaviour in healthy men.

1.5.5 Body composition

Since the chemical synthesis of testosterone in the 1930's there has been considerable interest in exploiting the anabolic effects of androgens. In the 1950's, Kochakian demonstrated the nitrogen retaining effects of testosterone in castrate males of many animal species. In the general population, reduced circulating androgen levels correlate with changes in body composition (Seidell *et al.*, 1990). Recently, there has been a resurgence of interest in the effects of androgens on body composition. Specifically, this has been associated with the aim of treating hypogonadal and elderly men, sarcopenia associated with HIV and also with the abuse of anabolic steroids in enhancing performance in athletes. The investigation of the effects of exogenous androgens on body composition in eugonadal subjects is of importance in the context of this thesis as they remain an essential component of male contraceptive regimes. The effects of testosterone on body composition on men has been investigated in

hypogonadal men, the elderly, during supraphysiological testosterone administration in male contraception, and in sarcopenic states such as HIV (Bhasin *et al.*, 1998).

Studies in hypogonadal men are in general agreement that testosterone replacement increases fat-free mass (FFM). Treatment of 18 hypogonadal men with transdermal testosterone delivering approximately 6mg/day and attaining normal testosterone concentrations by 3 months resulted in a mean increase in the FFM of 3.1kg (Snyder *et al.*, 2000). Similarly, the administration of various testosterone esters to hypogonadal men has persistently resulted in an increase in the FFM (Brodsky *et al.*, 1996; Katznelson *et al.*, 1996; Bhasin *et al.*, 1997) using different methods of measuring body composition including bio-electrical impedance, underwater weighing and DXA scanning. The percentage body fat is significantly higher in hypogonadal than eugonadal men. However the effects of testosterone replacement in hypogonadal men are more inconclusive either resulting in a decrease (Brodsky *et al.*, 1996; Katznelson *et al.*, 1996; Bhasin *et al.*, 1998) or no change (Bhasin *et al.*, 1997) in body fat. Similar to hypogonadal men, as men get older there is a decline in lean body mass and skeletal muscle with an increase in the % body fat (Steen, 1988). A study in 108 men comparing transdermal testosterone to placebo resulted in a significant increase in FFM and decrease in fat mass (FM) (Snyder *et al.*, 1999). Only two studies to date have investigated the effects of exogenous testosterone on body composition in the setting of male contraception. The first was on a cohort of men in the WHO study (World Health Organisation Task Force on Methods for the Regulation of Male Fertility, 1990) in Melbourne in the 1990's. Administration of a pharmacological dose of testosterone enanthate (200mg weekly) resulted in an increase in FFM of 9.6% and decrease in FM

of 16.2% at 6 months (Young *et al.*, 1993). More recently, the effects of a combination of the progestogen levonorgestrel (LNG) with TE were investigated. Similar to the previous study TE alone increased lean mass and decreased fat mass. However, this decline in fat mass was attenuated by combined progestogen-androgen regimen (Herbst *et al.*, 2003).

There may be several underlying mechanisms to explain the significant impact of androgens on body composition. Changes in FFM (detected using DXA scanning) include both lean body mass and total body water and may therefore reflect both. Holma *et al* (Holma, 1977) also demonstrated that over half the increase in body weight using anabolic steroids was accountable by an increase in total blood volume. The AR is present in skeletal muscle (Michel and Baulieu, 1980) and androgens increase protein synthesis in muscle, resulting in an increase in muscle mass/hypertrophy (Urban *et al.*, 1995). However, the muscle protein synthesis hypothesis does not explain testosterone-induced changes in fat mass. This testosterone-induced muscle hypertrophy is associated with a dose-dependent increase in muscle satellite cell and myonuclear cell number in healthy men (Sinha Hikim *et al.*, 2004). The AR is also present on adipose cells and testosterone enhances lipolysis (De Pergola *et al.*, 1990a; De Pergola *et al.*, 1990b; Xu *et al.*, 1990). Therefore testosterone may also directly affect adipose metabolism regulating lipolysis in a site-specific manner thus reducing fat mass. It has recently been proposed that the effects of testosterone on body composition relate to its primary site of action being on pluripotent stem cells committing their differentiation along the myogenic rather than adipogenic lineage (Bhasin *et al.*, 2003) which explains the reciprocal effects of testosterone on fat and muscle. There are of course other mechanisms of subtle regulation of body weight homeostasis that may be involved.

Leptin, the adipocyte-derived hormone may play a role and current evidence suggests that it may act at different levels of the hypothalamic-pituitary axis and may in fact directly inhibit testicular steroidogenesis explaining the link between hyperleptinaemia and low testosterone secretion in obese men (reviewed in (Tena-Sempere and Barreiro, 2002)). Conversely, testosterone administration in rats (Wu-Peng *et al.*, 1999) and its production under the influence of gonadotrophin treatment in hypogonadotrophin males (Kilciler *et al.*, 2002) leads to decreases in leptin levels. Thus by its interaction with the hypothalamic-pituitary axis, leptin may be involved in body weight haemostasis.

1.6. METABOLISM OF TESTOSTERONE & THE ROLE OF DHT

There are several possibilities for the metabolism of testosterone, often resulting in the production of essential biologically active metabolites. Aromatisation at the delta 4 bond gives rise to 17 beta-estradiol, 20-25% of which is produced by testis, the remainder by peripheral aromatisation. Reduction at this bond gives rise to 5 alpha-dihydrotestosterone (DHT). Both estradiol and DHT have essential biological roles acting through entirely different receptors in the cell. The action of androgens on the target cell are therefore dependent on these metabolic reactions. In the human, CYP 19 encodes for the aromatase cytochrome P₄₅₀ enzyme which is expressed in many tissues including the testis, liver, brain and hair follicles. Cases of complete aromatase deficiency have been reported (Morishima *et al.*, 1995; Carani *et al.*, 1997) resulting in infertility, macroorchidism, osteopenia, osteoporosis, hyperinsulinaemia, and reduced HDL-cholesterol levels. This demonstrates the functional importance of aromatase in

the male. Similarly, mutations in the ER gene result in hyperinsulinaemia and decreased bone density (Smith *et al.*, 1994). The amount of 17 beta-hydroxysteroid dehydrogenase in peripheral tissues determines the amount of active metabolite that is converted to the inactive estrone.

The conversion of testosterone to DHT by 5alpha-reductase is a key pathway in androgen metabolism and essential for the formation of the male phenotype during embryogenesis and for androgen-mediated growth of tissues such as the prostate. The testis produces approximately 100micrograms per day, the rest being converted from testosterone in other tissues. Testosterone and DHT bind to the same high affinity receptor in cell nuclei, DHT binding with greater affinity than testosterone (Wilbert *et al.*, 1983) probably due to a lower dissociation rate (Grino *et al.*, 1990). Despite binding to the same receptor, they appear to have differing physiological roles reflecting the tissue distribution of 5 α -reductase. The testosterone-receptor complex plays a major role in the stimulation of the Wolffian ducts during sexual differentiation and in the control of spermatogenesis and the DHT-receptor complex is involved in virilization and male sexual-maturation at puberty. This complex also demonstrates positive feedback on 5 α -reductase in pubic skin fibroblasts and the prostate (George *et al.*, 1991) leading to an increase in its production.

Two isotypes of the enzyme have been identified, 5 α -reductase type 1 and type 2 (review in (Russell and Wilson, 1994)). The type 2 isotype is the major genital enzyme, deficiency of which results in the clinical condition of male pseudohermaphrodis, previously known as 'pseudovaginal perineoscrotal hypospadias' (Andersson *et al.*,

1991; Wilson *et al.*, 1993). This condition results from an autosomal recessive inherited single gene defect for 5 α -reductase isotype 2, manifesting as ambiguous genitalia at birth with male internal genitalia. The investigation of families with this condition in the 1970's in Dallas (Walsh *et al.*, 1974) and the Dominican Republic (Imperato-McGinley *et al.*, 1974) with the common finding of a deficiency in 5 α -reductase type 2 culminated with the cloning of the genes encoding the isotypes (Andersson and Russell, 1990; Jenkins *et al.*, 1992) and the further investigation of their pathophysiological importance.

1.7. THE HYPOTHALAMIC-PITUITARY AXIS – THE REGULATION OF GONADOTROPHIN SECRETION BY TESTICULAR STEROIDS

Luteinising Hormone (LH) and Follicle Stimulating Hormone (FSH) are secreted by the gonadotrophs of the anterior pituitary gland in response to GnRH. GnRH is secreted from the terminal neurons of the medial preoptic area of the hypothalamus into the hypophyseal portal blood (Davidson and Bloch, 1969; Elde and Hökfelt, 1978; Silverman *et al.*, 1979). Portal catheterisation models in the sheep have demonstrated that the secretion of gonadotrophins is pulsatile in nature and their secretion closely parallels that of GnRH (Clarke and Cummins, 1982). The demonstration of this in humans *in vivo* has not been possible, therefore with this limitation many studies assume that the secretion in humans is similar to that in animal studies. The investigation of feedback control of gonadotrophin secretion use pulse frequency and amplitude as a means of assessing hypothalamic and pituitary secretion. However,

caution must be observed in their interpretation as this may reflect both hypothalamic and pituitary sites of action. The following discussion will focus on the regulation exerted by testosterone and oestradiol.

The feedback effects of the testicular steroids have been investigated through administration of the steroid itself (although often at pharmacological doses), blockade of the endogenous receptor or by inhibiting the reduction of testosterone to DHT and aromatisation of testosterone to oestradiol. Both testosterone and DHT inhibit gonadotrophin secretion (Sherins and Loriaux, 1973; Santen, 1975). Many studies have reported the site of their action to be the hypothalamus from the observed decrease in detected LH pulse frequency resulting from a decrease in pulsatile GnRH secretion following their administration (Winters *et al.*, 1979; Matsumoto and Bremner, 1984; Finkelstein *et al.*, 1991a). However, pituitary feedback has been demonstrated with a decline in LH pulse amplitude in GnRH deficient men following testosterone infusion (Winters *et al.*, 1979; Matsumoto and Bremner, 1984; Finkelstein *et al.*, 1991a). Similarly, in men with hypogonadotrophic hypogonadism normalised with a physiological dose of GnRH, testosterone blunts the LH response to GnRH demonstrating a GnRH independent mechanism, perhaps through direct pituitary inhibition (Sheckter *et al.*, 1989). Furthermore, selectively blocking the AR with flutamide thereby investigating the endogenous effects of androgens, not only results in an increase in LH pulse frequency (Urban *et al.*, 1988) but also LH amplitude (Urban *et al.*, 1988; Veldhuis *et al.*, 1992). Inhibition of endogenous 5 alpha-reductase production with finasteride has resulted in no effect on basal or GnRH-stimulated gonadotrophin concentrations, although there was a slight increase in the overall testosterone

concentrations (Rittmaster *et al.*, 1992). Other studies have reported a varying response from DHT infusions: suppression of LH concentrations (Santen, 1975), augmentation of the LH response to GnRH (Winters *et al.*, 1979), or no change in gonadotrophin levels at all (Bagatell *et al.*, 1994a).

Similarly the investigation of the role of oestrogen on feedback control of gonadotrophin secretion, has been approached by infusion of the steroid itself (Santen, 1975; Finkelstein *et al.*, 1991b; Bagatell *et al.*, 1994a; Hayes *et al.*, 2000), blockade of the endogenous receptor by anti-oestrogens such as clomiphene (Boyar *et al.*, 1973; Winters *et al.*, 1979; Winters and Troen, 1985) or tamoxifen (Spijkstra *et al.*, 1988), and by inhibition of the aromatisation of testosterone (Finkelstein *et al.*, 1991a; Hayes *et al.*, 2000; Hayes *et al.*, 2001). Oestradiol infusion has been demonstrated to suppress the secretion of gonadotrophins through a reduction in pulse amplitude and LH responsiveness to GnRH with no effect of pulse frequency (Kulin and Reiter, 1972; Sherins and Loriaux, 1973; Santen, 1975; Winters *et al.*, 1979; Gooren, 1989) implying a pituitary site of action. However, administration of anti-oestrogens to normal men results in an increase in pulse frequency (Boyar *et al.*, 1973; Santen and Ruby, 1979; Winters and Troen, 1985; Veldhuis and Dufau, 1987; Spijkstra *et al.*, 1988) consistent with a hypothalamic site of oestrogen feedback. Also, in animal models the administration of oestrogen to castrate sheep results in a decrease in LH concentrations by decreasing LH pulse frequency (Schanbacher, 1984; Scott *et al.*, 1997). Administration of the aromatase inhibitor, testolactone, inhibits the effects of testosterone on the hypothalamic-pituitary axis implying that these effects are partly dependent on the aromatisation of testosterone (Finkelstein *et al.*, 1991a). However

aromatase has also been shown to have anti-androgenic properties in binding to the androgen receptor (Vigersky *et al.*, 1982). Many of the previous studies can be criticised on several points. Using pharmacological doses of oestrogen may not reflect normal physiological feedback. Furthermore, in normal men endogenous feedback confounds the feedback mechanisms under investigation. Hayes *et al* used a hypothalamic clamping model in men with idiopathic hypothalamic hypogonadism normalising their axes with long-term GnRH, in parallel with normal men. By selectively inhibiting aromatase with anastrozole, endogenous oestrogen production was blocked, and mean LH levels rose with an increase in both LH frequency and amplitude (Hayes *et al.*, 2000). This is consistent with endogenous oestrogen in the human male having a dual site of action, both at the hypothalamus decreasing GnRH pulse frequency, and at the pituitary reducing the responsiveness to GnRH.

In addition to the observed difference in effect of the different testicular steroids on LH at physiological and pharmacological concentrations, they have a different effect on FSH. Earlier studies demonstrated that pulsatile GnRH secretion was needed for LH but not for FSH secretion (Fauser *et al.*, 1983) and increasing the GnRH pulse generator increased LH but not FSH secretion (Spratt *et al.*, 1987; Sauder *et al.*, 1988). In addition, Urban *et al* demonstrated that DHT and testosterone had no effect on FSH pulse frequency while affecting that of LH (Urban *et al.*, 1991). The increase in FSH on selective estrogen withdrawal (with selective aromatase blockade) was no greater than during medical castration (removing both testosterone and oestrogen) suggesting that the influence of testosterone on FSH is largely dependent on aromatisation to oestrogen (Hayes *et al.*, 2001). In addition to regulation of gonadotrophins by testicular steroids,

there is an additional level of complexity with FSH as it is also regulated by non-steroidal factors; inhibin B from the testis and autocrine/paracrine regulation at the level of the pituitary with the influence of activin and follistatin (Ying, 1988).

1.8 INHIBIN

Control of gonadotrophins is also mediated by the peptide hormone inhibin. The concept of a testicular protein hormone regulating FSH secretion was initially proposed by McCullagh in 1932 and is now extensively characterised. Inhibin is a dimer composed of an α -subunit and β A (Inhibin A) or β B (Inhibin B) subunit. Following the development of the dimeric assay it has been demonstrated that Inhibin B is the relevant type in the human male whereas Inhibin A is undetectable (Anawalt *et al.*, 1996; Illingworth *et al.*, 1996). The B subtype is present in both fetal and adult males and suppresses the secretion of FSH.

Castration in animals results in a significant decline in its secretion, demonstrating its gonadal source. There is general agreement that the Sertoli cell is the predominant site of inhibin production (Bergh and Cajander, 1990; Majdic *et al.*, 1997; Anderson *et al.*, 1998). Both subunits have been localised to the fetal and adult Sertoli cell (Bergh and Cajander, 1990; Majdic *et al.*, 1997; Anderson *et al.*, 1998; Andersson *et al.*, 1998a). Purified cultures of human Sertoli cells secrete inhibin B and this is increased in the presence of germ cells (Carreau, 1995) suggesting interaction between the two cell types. Actual manipulation of Sertoli cell number in animal models both positively and negatively results in parallel changes in inhibin concentrations (Ramaswamy *et al.*,

1999; Sharpe *et al.*, 1999). Both α and β B subunits have been also found in Leydig cells of the adult human testis (Anderson *et al.*, 1998; Andersson *et al.*, 1998b). This is consistent with them being able to produce Inhibin B, as Leydig cells are sources of both the α and the β subunits. A recent study has investigated the response of the Leydig cell to hCG; as expected a rise in blood testosterone follows with no change in the levels of inhibin B however a rise in the pro- α C subunit was observed (Kinniburgh and Anderson, 2001). Therefore, although much of the evidence is indirect, close coordination of function between the different cell types of the testis in secreting inhibin B might be postulated. Subunits are also produced locally at the level of the pituitary (de Kretser *et al.*, 2001).

The control of inhibin secretion is exerted both locally and by circulating feedback exerted by testosterone and inhibin (de Kretser *et al.*, 2001). Following the development of the first specific assay for inhibin B (Groome *et al.*, 1996) there has been considerable investigation of the changes in inhibin in many pathophysiological states. This has significantly increased our understanding of the complex interplay between FSH, inhibin and spermatogenesis. Prior to this, there have been considerable data demonstrating the dependence of inhibin secretion on FSH both in vivo and in vitro. Following hypophysectomy in the rat, the secretion of inhibin decreases and this is restored by FSH but not testosterone (Au *et al.*, 1985). Similarly in primates, GnRH antagonists suppress the secretion of inhibin, which is again restored by FSH (Weinbauer *et al.*, 1991).

In the human male, serum inhibin B levels (as well as their relationship with FSH) vary throughout reproductive life with an apparent switch in inhibin regulation occurring at puberty. While Sertoli cell proliferation and FSH are major determinants in childhood, germ cells i.e. spermatogenic status also become important influences in the adult. In the newborn inhibin levels are low in cord blood, rising to levels that are higher than the adult by 3-6 months in the neonate (Andersson *et al.*, 1997; Andersson *et al.*, 1998b). This may mirror activation of the hypothalamic-pituitary-testicular axis at this stage and the neonatal proliferation of Sertoli cells. Following this early postnatal rise levels fall to a nadir by 3-6 years during which time serum FSH levels are low, perhaps suggesting early development of negative feedback. Basal inhibin B levels increase under the influence of FSH in early pubertal stages with the last wave of Sertoli cell development. However by Tanner stages G3/G4, FSH and Inhibin B levels correlate negatively, suggesting that the negative feedback regulation loop is fully established (Andersson *et al.*, 1997; Raivio and Dunkel, 1999; Raivio *et al.*, 2000). Therefore the main regulatory factor in inhibin secretion, FSH, seems to switch at puberty. In the adult male the production of inhibin B is not only dependent on the presence of FSH but is also directly proportional to the amount of spermatogenesis. This is supported by the direct correlation between sperm count and serum inhibin B concentrations (Pierik *et al.*, 1998). Under conditions when this falls such as in Sertoli cell only syndrome or irradiation, inhibin B falls and FSH increases (Andersson and Skakkebaek, 2001). Clinical studies in the adult demonstrate an inverse relationship between inhibin B and FSH both in healthy men and in men with testicular disorders (Anawalt *et al.*, 1996; Illingworth *et al.*, 1996; Wallace *et al.*, 1997).

Following the demonstration that Inhibin B is the relevant dimer in man, there have been several investigations of its function in a variety of pathophysiological settings including infertility, hypogonadotrophic hypogonadism, ageing, radiation, chemotherapy and hormonal male contraception. Studies in men treated with hormonal contraceptives have led to conflicting results. The administration of TE alone has resulted in a rapid and progressive decline in serum inhibin B concentrations (Andersson *et al.*, 1997; Zhengwei *et al.*, 1998). In combination with the gestogen levonorgestrel, TE administration for 6 months resulted in a significant decline in inhibin B (Anawalt *et al.*, 1999). However, in a further study using transdermal testosterone, no change was observed (Büchter *et al.*, 1999). Similarly, administration of oral desogestrel plus testosterone pellets (Martin *et al.*, 2000b; Kinniburgh *et al.*, 2002) or etonogestrel implants with testosterone pellets (Anderson *et al.*, 2002a) results in no change in serum inhibin B levels. In contrast, seminal plasma levels become undetectable with significantly reduced serum pro- α C concentrations indicating the close relation between gonadotrophins and pro- α C concentrations. The reason for the disparity between serum and seminal plasma inhibin B is unclear, and may reflect loss of the integrity of inter-Sertoli cell junctions under these conditions. The close correlation between seminal plasma Inhibin B and sperm concentrations clearly indicate that it is a closer marker of seminiferous epithelium activity. The underlying reason for the conflicting results relating to Inhibin B with male hormonal contraceptive regimes remains unclear, although may relate to the type of combination, or the duration of treatment.

In summary, the testis is controlled from fetal life by a complex feedback system involving the hypothalamus, anterior pituitary and substances secreted by the testis itself. These include testosterone, inhibin B and oestradiol, also produced from the peripheral aromatisation of testosterone. Testosterone has important effects in regulating the seminiferous epithelium as well as diverse systemic actions on libido, body weight haemostasis, serum lipoprotein regulation and prostate growth and development. LH stimulates testicular steroidogenesis and FSH promotes the process of spermatogenesis, however the exact mechanisms are incompletely understood. The suppression of gonadotrophins is an obvious target for male contraception in attempting to inhibit spermatogenesis. The following discussion will focus on this area and the attempts to date to develop a male hormonal contraceptive.

1.9 MALE HORMONAL CONTRACEPTION

With one third of the world's population relying on male methods of contraception, namely condoms, coitus interruptus, abstinence and vasectomy (United Nations, 1994), there is a need to expand choice for couples relying on a male method. The concept of male hormonal contraception is by no means new; as early as the 1930's reversible inhibition of spermatogenesis with little effect on libido was demonstrated (McCullagh and McGurl, 1939), by administration of testosterone. Since then many attempts have been made at providing a safe, reversible means of inducing azoospermia, without any long-term adverse effects on health. In addition to issues of safety and efficacy, acceptability and speed of onset of action require to be considered. However, progress over the decades has been slow and pharmaceutical industry interest and funding has been limited. Furthermore, our incomplete knowledge of male reproductive physiology has limited the potential targets for inhibition of fertility. These targets can be classified as follows:-

1. Pre-testicular: withdrawal of gonadotrophin support of the testis (LH and FSH).
2. Testicular: interfering with spermatogenesis at the level of the seminiferous tubules.
3. Post-testicular: disrupting sperm maturation and transport, particularly at the level of the epididymis.

The testicular and post-testicular approaches have several theoretical advantages, including specificity of action to the reproductive system, testosterone replacement is

not required, and rapidity of onset. Difficulties remain in identifying aspects of testicular or epididymal function without toxicity. The most widely tested compound in this respect has been gossypol, derived from cotton seed. While this agent resulted in a very high prevalence of azoospermia, this was in many cases irreversible and there were other side effects including hypokalaemia (Waites *et al.*, 1998). It is however likely that with increasing understanding of the molecular basis of spermatogenesis and sperm function that an appropriate target will be found. Recently identified molecules which may be potential targets include ion channels specifically expressed by sperm (Ren *et al.*, 2001), and molecules involved in the interaction between germ cells and Sertoli cells (Akama *et al.*, 2002). The focus of the remainder of this discussion will be on the pre-testicular, hormonal, approach to male contraception as it is this area which forms the basis of this thesis

1.9.1 Hormonal approaches

The revolution in female contraceptive provision enabled by advances in steroid chemistry in the second half of the last century have focused attention on the analogous targets in the male, i.e. the administration of exogenous hormone to inhibit the production of gonadotrophins at the level of the hypothalamus and pituitary gland. Inhibition of both FSH and LH is required for adequate inhibition of spermatogenesis but the fall in testicular steroidogenesis will result in symptoms of hypogonadism and in the longer term decreased bone mass, muscle bulk and deficient haematopoiesis. Therefore, androgen replacement remains an essential component of all male hormonal contraceptive regimes. While supraphysiological doses of testosterone will suppress spermatogenesis by inhibiting gonadotrophin secretion, an approach demonstrated to be

an effective contraceptive (discussed below), it may also paradoxically support spermatogenesis, although this has been much more clearly demonstrated in rodents than in man. Therefore, the aim is to provide sufficient testosterone to suppress gonadotrophin production and prevent hypogonadism without stimulating spermatogenesis. The co-administration of a second agent such as a progestogen or gonadotrophin releasing hormone (GnRH) analogue makes this much more readily achievable. While earlier studies using testosterone administration alone did not prove to be a feasible contraceptive, much valuable data has come from studies using this approach.

1.9.2 Testosterone enanthate: proof of concept

The degree of reduction in sperm concentration from the normal range of 20 to 200 million/ml required to achieve acceptable contraceptive efficacy is uncertain. Indeed, pregnancies have been seen in partners of men in contraceptive studies whose sperm concentration was suppressed to below 1×10^6 /ml although exposure was for many months prior to conception (Barfield *et al.*, 1979). This was specifically investigated in two studies carried out by World Health Organisation (WHO) designed to investigate the degree of spermatogenic suppression required for contraceptive efficacy. These studies used a prototype regimen of testosterone enanthate (TE) administered weekly and were based on encouraging results from the 1970's (Steinberger and Smith, 1977; Swerdloff *et al.*, 1979), when over half Caucasian subjects attained azoospermia, the remainder becoming oligozoospermic.

The first WHO study investigated contraceptive efficacy in subjects once they had achieved azoospermia, the subsequent study enlarged on this to include all men whose sperm concentrations had fallen below $3 \times 10^6/\text{ml}$, both based on weekly administration of 200mg TE i.m. There were no pregnancies per 230 person-years in the azoospermic group but a small but significant number of pregnancies in the oligozoospermic group (4 pregnancies proportional to sperm output, with 49.5 years of exposure). These data gave an overall pregnancy rate of 1.4 per 100 person-years (95% CI 0.4 – 3.7) (World Health Organisation Task Force on Methods for the Regulation of Male Fertility, 1990, 1996). The pregnancy rate in those men with sperm concentrations between $0.1 - 3 \times 10^6/\text{ml}$ was 8.1 per 100 person-years (95% CI 2.2-20.7). 98% of subjects actually entered the 12 month efficacy phase of the study, achieving concentrations of less than 3×10^6 within 6 months of treatment. These studies served as a clear demonstration that steroid induced suppression of spermatogenesis is a possibility for a contraceptive method in that it is effective and entirely reversible. However, the significant risk of pregnancy in the oligozoospermic group emphasises the need to develop a method achieving more uniform azoospermia. Supraphysiological testosterone levels however, resulted in potentially adverse metabolic effects, particularly a decrease in high-density lipoprotein cholesterol (HDL-C), a risk factor for ischaemic heart disease (Bagatell *et al.*, 1992; Anderson *et al.*, 1995; Meriggiola *et al.*, 1995), although this was not seen in the Chinese subjects (Wu *et al.*, 1996). These important studies therefore confirmed the concept of hormonal suppression for contraceptive efficacy and that the development of long-acting testosterone preparations was required in order to exploit this demonstration of proof of concept.

1.9.3 Ethnic polymorphism in response

A striking feature from these studies is the ethnic heterogeneity in the degree of suppression of spermatogenesis with Asian men consistently showing a higher prevalence of azoospermia than Caucasian men, following exogenous sex steroid administration (World Health Organisation Task Force on Methods for the Regulation of Male Fertility, 1995). The underlying mechanisms have been extensively investigated and at present remain unclear (Handelsman *et al.*, 1995; Anderson *et al.*, 1996). It has been demonstrated that higher 5 α -reductase activity in non-responders may be supporting spermatogenesis due to residual intra-testicular DHT (Anderson *et al.*, 1996). There may be differing sensitivity in the hypothalamic–pituitary axis: following administration of exogenous testosterone, a greater reduction in gonadotrophin pulse amplitude, at lower doses of testosterone, is observed in Asian men in comparison to Caucasians (Wang *et al.*, 1998a). The metabolic clearance rates of androgens do seem to differ between Asian and Caucasian men, however production rates of testosterone may be lower in Asian men living in Asia, perhaps suggesting some dietary or environmental influence (Santner *et al.*, 1998). Morphological studies have demonstrated that Asian men may have a lower potential for spermatogenesis with smaller testis and seminiferous tubule volume, and a lower number of Sertoli cells (Johnson *et al.*, 1998). Furthermore, an increased basal apoptotic rate in germ cells of Asian man may contribute to this ethnic polymorphism in response (Sinha Hikim *et al.*, 1998). A recent study (Yu and Handelsman, 2001) has investigated whether functional aspects of androgen action may contribute to the variable susceptibility to hormonal induced azoospermia in men receiving testosterone enanthate. However, neither pharmacogenetic polymorphisms in the AR (CAG and GGC repeats) and the *CYP3A4*

gene encoding hepatic cytochrome p450, were associated with variation in susceptibility to azoospermia. Thus, evidence to date suggests that there is an ethnic or geographic difference in testes responsiveness to gonadotrophin suppression with administration of exogenous steroid. However, the exact underlying mechanisms remain unclear. We will consider these mechanisms further in the course of this thesis.

1.9.4 Androgen/Progestogen combinations

Progestogens are potent inhibitors of gonadotrophins in men as in women thus in combination with testosterone allow lower doses of each steroid to be given. Consequently androgen associated side effects and ongoing androgen-supported spermatogenesis are avoided. An advantage is their availability: they have been in use for several decades as components of female hormonal contraception and the range of existing products can be administered orally, intramuscularly or by subcutaneous implants.

Depot medroxyprogesterone acetate (DMPA) has been widely investigated as the progestogen component. In a series of studies in the 1970s, over 100 men were given monthly DMPA injections in combination with monthly TE 100 to 250mg for up to 16 months (Alvarez-Sanchez *et al.*, 1977; Brenner *et al.*, 1977; Frick *et al.*, 1977b, a; Melo and Coutinho, 1977). Although azoospermia was achieved in half the subjects, contraceptive efficacy was poor with several pregnancies resulting. Side-effects from this regime included weight gain, gynaecomastia and a transient decline in HDL-C. Similar to androgen-only regimes ethnic polymorphism in spermatogenic response is evident with androgen-progestogen combinations. A higher prevalence of azoospermia

was found in studies in Indonesian men, with 97% achieving azoospermia in comparison to 60-67% of Caucasian subjects, using DMPA with 19-nortestosterone or TE (World Health Organisation Task Force on Methods for the Regulation of Male Fertility, 1993). The high prevalence of azoospermia in Indonesian men with testosterone-only regimens however means that the contribution of the DMPA in that study is unclear. Doubling the dose of TE did not increase efficacy of this regime in Caucasian men (Faundes *et al.*, 1981). However, administration of testosterone pellets (800mg) with 300mg DMPA (single administration only) resulted in a high incidence of azoospermia with 9/10 men becoming azoospermic compared to 4/10 with testosterone alone (Handelsman *et al.*, 1996). This approach was further investigated in the first male contraceptive efficacy study using a combined progestogen/androgen approach. Fifty-five healthy men received 300mg DMPA 3 monthly and 800mg T pellets, initially every 6 months, changing to 4 monthly during the course of the study due to symptoms of hypoandrogenism. 94% of subjects entered a 12 month efficacy phase, with a threshold of suppression of spermatogenesis to $<1\text{M/ml}$, resulting in no pregnancies in 426 person-months of contraceptive exposure (Turner *et al.*, 2003). DMPA has recently been investigated in combination with the testosterone undecanoate (TU) as a convenient 8 weekly injection, in a Phase I clinical study in Chinese men. Consistent azoospermia or severe oligozoospermia was achieved in all subjects treated with TU 1000mg and DMPA (150 or 300mg) every 8 weeks, with no serious long-term effects (Gu *et al.*, 2004).

Levonorgestrel has also been widely investigated in combination with testosterone. Results from earlier studies using a combination of oral levonorgestrel with TE were

encouraging. Administration of 250 to 500µg levonorgestrel with low dosage TE (200mg/month) suppressed sperm concentration in half the subjects below $5 \times 10^6/\text{ml}$ but no subjects reached azoospermia (Foegh *et al.*, 1980). Recent randomized controlled trials with more frequent testosterone dosing not only showed increased suppression of spermatogenesis but also demonstrated that the levonorgestrel-TE combination was superior to using androgen alone (Bebb *et al.*, 1996). Administration of 500µg levonorgestrel with 100mg TE weekly resulted in 94% of subjects becoming azoospermic or severely oligozoospermic in comparison to 61% in the TE group alone. In addition, the time to onset of azoospermia was more rapid in the combined group. However, similar to previous progestogen combinations there was a significant reduction in HDL-C of approximately 20% and subjects also experienced weight gain. Subsequent studies lowering the dose of levonorgestrel to 125µg orally, resulted in a slight reduction in suppression of spermatogenesis (78% compared to 89% with 500µg), but with reduced weight gain and HDL-C suppression (Anawalt *et al.*, 1999). A recent study further investigated the changes in weight at this dose of levonorgestrel (125µg) demonstrating that when administered alone an increase in fat mass is observed. However, given in combination with TE, although resulting in an increase in lean body mass there is no change in fat mass (Herbst *et al.*, 2003) and therefore the combination have a more favourable effects on body composition profile.

Another approach has recently been reported combining a long acting depot injection of testosterone undecanoate (TU) with the progestogen norethisterone (NET) enanthate. NET enanthate has been established as a depot contraceptive in women (Fotherby *et al.*, 1984), currently licensed in the UK for short-term use. NET enanthate has been shown

to have a profoundly suppressive effect on gonadotrophins in men (Kamischke *et al.*, 2001) and demonstrates some unique biochemical properties. NET binds to the androgen receptor with 45% the affinity of testosterone resulting in androgenic activity of about 10% that of testosterone (Ojosoo and Raynaud, 1983), which may be of further benefit in male contraception. It undergoes 5 α -reduction to 5 α -NET, enhancing its relative binding affinity for the androgen receptor but unlike DHT, paradoxically diminishing its androgenic potency in target organs (Lemus *et al.*, 1997) such as the prostate. Used as a contraceptive agent in men, the injection intervals were conveniently equal for both steroids (6 weekly) and results from the combination were superior to TU alone with 13 out of 14 compared to 7 out of 14 men becoming azoospermic (Kamischke *et al.*, 2001).

One of the most promising results to date using this combination has been observed using the synthetic progestogen desogestrel (Wu *et al.*, 1999; Anawalt *et al.*, 2000). In one study eight out of eight men became azoospermic with oral desogestrel 300 μ g daily in combination with 50mg TE intramuscular weekly (Wu *et al.*, 1999). This study also demonstrated the narrow dose-response relationship with this combination: either decreasing the dose of desogestrel to 150 μ g or a higher dose of testosterone (100mg/week) resulted in a lower apparent incidence of azoospermia although the groups were of small size. In a further study, 94% of men became azoospermic on receiving 150 or 300 μ g desogestrel in combination with 100mg TE weekly (Anawalt *et al.*, 2000). Once again, the narrow dose-response was evident with reduction of the dose of testosterone reducing levels of suppression. Desogestrel also had a dose-dependent effect on lowering of HDL-C and weight gain. In a dose-finding study,

300µg desogestrel with testosterone pellets resulted in greater spermatogenic suppression than 75 or 150µg (Martin *et al.*, 2000b). It has been recently demonstrated that this combination of 300µg desogestrel and 400mg T pellets 12 weekly, resulted in azoospermia in all subjects investigated in both Scotland and Shanghai (Kinniburgh *et al.*, 2002). As with other testosterone/gestogen preparations, weight gain and HDL-C suppression were reported and dose-dependency of gonadotrophin suppression by desogestrel. This combination has resulted in effective suppression of spermatogenesis in 2 populations from Africa, albeit to a lesser extent, with an overall prevalence of azoospermia of 74% (Anderson *et al.*, 2002b).

Long-acting progestogen implant preparations offer another attractive method of delivery. In addition to not being user-dependent, a more sustained release preparation may allow dosage reduction and avoid fluctuating serum steroid levels that may result from oral treatments. Few data are however available. Administration of levonorgestrel implants in combination with injectable testosterone undecanoate (250mg/month) resulted in 6 out of 18 Chinese subjects becoming azoospermic (Gao *et al.*, 1999). A further study investigated levonorgestrel implants (Norplant II ®) in combination with transdermal testosterone patches and TE, also comparing to T patches alone and oral LNG with T patches (Gaw Gonzalo *et al.*, 2002). Results were disappointing with severe oligozoospermia only in <60% of subjects with the combination of T patch and Norplant. Both this group and the oral LNG/patch were less efficacious than previous combinations with TE and LNG (Anawalt *et al.*, 1999). Whilst achieving equivalent serum concentrations of LNG in this study, the lesser suppression probably resulted from lower testosterone concentrations with the patch preparation in comparison with

weekly TE injections. Although Norplant II with TE were most effective, suppressing spermatogenesis to severe oligozoospermia in 100% and to azoospermia in 93% of subjects, this combination would still necessitate weekly injections. Results have been very encouraging with Implanon® (Organon, NV) which releases etonogestrel, the active metabolite of desogestrel. Administration of 1 implant similar to that used in women, in combination with testosterone pellets resulted in 64% azoospermia and (10/14) 71% with concentrations of $<1\text{M/ml}$. However, increasing the dose to 2 implants greatly increased suppression of spermatogenesis, with 13/14 (92%) of men achieving sperm concentrations $<0.1 \times 10^6/\text{ml}$ and 75% achieving azoospermia (Anderson *et al.*, 2002a).

To date several androgen/gestogen combinations have been used with a significant improvement in efficacy from previous androgen-only approaches. However, extra-testicular side-effects, namely on lipid metabolism and with weight gain remain a problem which future regimes will aim to minimize. This approach will be considered further in the three clinical studies in this thesis using both oral and long acting implants.

1.9.5 Androgen/Anti-androgen combinations

Administration of an anti-androgen might appear inappropriate in a hormonal regimen that requires maintenance of peripheral androgen action. However inhibition of the action of residual intratesticular concentrations of testosterone may increase efficacy in attaining azoospermia. The measurement of the testicular steroid epi-testosterone during gonadotrophin withdrawal supports ongoing testicular steroidogenesis, albeit at

low levels (Dehennin and Matsumoto, 1993; Anderson *et al.*, 1997a). In models of the LH (LuRKO) knockout mouse quantitatively normal spermatogenesis is achieved despite very low intratesticular T concentrations. Post-meiotic spermiogenesis was however blocked by administration of flutamide, indicating a crucial role for residual low testicular testosterone (Zhang *et al.*, 2003). In the human male, under conditions of gonadotrophin withdrawal, testicular DHT concentrations are maintained despite markedly reduced intra-testicular T concentrations (Mc Lachlan *et al.*, 2002). Therefore 5 α -reductase may 'amplify' these low T levels in the testis and indeed this enzyme may be up-regulated under these conditions (Pratis *et al.*, 2000). Therefore antagonising this potentially important effect of residual intratesticular androgen by administration of an anti-androgen as part of a contraceptive regime may enhance spermatogenic suppression.

In this context, cyproterone acetate (CPA), an anti-androgen and progestogen, has been investigated as a potential male contraceptive. Administration of CPA alone at a dose of up to 100mg/day resulted in a moderate reduction in sperm production, but a marked suppression in androgen levels and libido (Wang and Yeung, 1980) therefore administered alone, it would not be suitable as a male contraceptive. Subsequent studies in India of CPA in combination with TE, rather than CPA alone, resulted in azoospermia in the majority of subjects with no major adverse effects (Roy, 1985). This combination has been recently reinvestigated, 5 patients in each group receiving 50mg or 100mg CPA daily orally with 100mg TE weekly, or TE alone at the same dose (Meriggiola *et al.*, 1996b). The adjuvant benefit of CPA was apparent with all subjects in those groups attaining azoospermia whereas only 3/5 of the testosterone alone group

exhibited similar suppression. Subsequent dose-finding studies demonstrated high efficacy of CPA at doses greater than 25mg/day with no adverse effect on blood lipid profile (Meriggiola *et al.*, 1998). However, there was a dose-dependent suppression of haemoglobin and haematocrit reflecting its anti-androgenic effect. With the aim of developing a 'male pill', further studies investigated a further decrease in CPA dose to 12.5mg combined with oral testosterone undecanoate 80 mg twice daily (Meriggiola *et al.*, 1997). Results were however disappointing with azoospermia being achieved in only 2 out of 8 subjects. Again this emphasizes the narrow therapeutic index of male contraceptive regimes and the fine balance between the anti-androgenic properties of CPA and the androgenic effects of testosterone.

1.9.6 The potential of 5 α -reductase inhibition

The enzyme 5 α -reductase converts testosterone to DHT. DHT has greater affinity for the androgen receptor and thus acts as an amplifier of testosterone action in tissues in which it is highly expressed such as the prostate. Two isoforms of the enzyme have been identified (Russell and Wilson, 1994). Under physiological conditions, intratesticular testosterone concentrations are high and are presumed to saturate the androgen receptor. However when endogenous production is low, conversion to DHT may become an important factor in the maintenance of spermatogenesis. 5 α -reductase activity has been demonstrated in the human testis (Payne *et al.*, 1973; Rivarola *et al.*, 1973). However, evidence is scant regarding the nature, regulation and isoform of testicular 5 α -reductase in the adult male. mRNA levels and enzymatic activity are very low in the human testis although the presence of the type 2 enzyme isoform is suggested (Thigpen *et al.*, 1993). In contrast, type 1 is the predominant isoform in the rat testis



(O'Donnell *et al.*, 1996). It has been suggested that differences in 5 α -reductase activity may underlie the heterogeneity of response to TE, explaining why some individuals maintain oligozoospermia (Anderson *et al.*, 1996). Parallels have also been drawn between 5 α -reductase activity in different ethnic groups (Lookingbill *et al.*, 1991; Ross *et al.*, 1992) with lower levels of activity in Asian populations which may relate to the differing response between Caucasian and non-Caucasian populations discussed above. The importance of 5 α -reductase has been demonstrated in adult rats with the inhibition of 5 α -reductase leading to an increased requirement of testosterone to support spermatogenesis, implying that conversion of testosterone to DHT may permit ongoing spermatogenesis when intratesticular testosterone levels are reduced (O'Donnell *et al.*, 1996). However, the extent to which 5 α -reductase supports spermatogenesis in humans is less clear. In a recent clinical study, administration of finasteride, a 5 α -reductase type 2 inhibitor, did not enhance the rate or degree of suppression of spermatogenesis in males on administering a progestin/testosterone combined contraceptive regime (Kinniburgh *et al.*, 2001). Similarly, in men failing to suppress within 3 months of treatment with testosterone implants there was no enhancement of suppression of spermatogenesis when finasteride was added (McLachlan *et al.*, 2000). Although the effect of co-administration with an inhibitor of the type 1 isoform or dutasteride which inhibits both isoenzymes has not been investigated, at present no additional benefit is evident from 5 α -reductase inhibition in a contraceptive regimen.

1.9.7 Androgen/oestrogen combinations

Evidence is now emerging to support an important role for oestrogen in spermatogenesis. In the oestrogen receptor- α (ER- α) knockout mouse, the seminiferous tubules are dysfunctional and the epididymis is unable to support sperm maturation with consequent infertility (Lubahn *et al.*, 1988; Korach *et al.*, 1996; Hess *et al.*, 1997). Individuals with oestrogen receptor mutations and aromatase-deficiency illustrate the importance of aromatisation in the physiology of the male (Smith *et al.*, 1994; Morishima *et al.*, 1995). A male reported to have a mutation in CYP19 encoding aromatase was infertile (Carani *et al.*, 1997). However, his brother with a normal CYP19 gene was also infertile and thus familial occurrence limits the interpretation of the impact of this mutation on spermatogenesis. Oestrogen blockade may therefore become a potential target for contraception. However, this would have to be in a selective manner in order to avoid associated adverse effects such as osteoporosis and hyperlipidaemia.

An alternative basis for the use of oestrogen in male contraception is the demonstration that aromatisation is crucial for mediating the feedback effect of testosterone on FSH secretion (Hayes *et al.*, 2000). The enhanced suppression of spermatogenesis from the addition of low dose oestradiol to testosterone in non-human primates was demonstrated in a series of studies in the 1970's (Ewing *et al.*, 1977) and has recently been confirmed in humans (Handelsman *et al.*, 2000). However, the therapeutic margin before the occurrence of oestrogenic side effects was narrow and the levels of suppression achieved suboptimal for an acceptable contraceptive method. The administration of oestrogen also raises safety concerns with the hypothetical risk of arterial

thromboembolism. It seems unlikely at present that this combination will be used, however these data emphasize the importance of aromatization of any synthetic androgen used in this context.

1.9.8 GnRH agonists and antagonists

GnRH agonist analogues have become established therapies in a wide range of hormone-dependent diseases. In combination with an androgen, suppression of gonadotrophin secretion and consequent suppression of spermatogenesis is a further potential application. The more specific action than with other steroids such as progestogens may diminish adverse systemic effects and lower the total dose of steroid required. However, in general results from clinical studies involving administration of GnRH agonists subcutaneously (D-Trp⁶, buserelin and nafarelin) with varying doses of TE have been disappointing with failure of complete suppression of spermatogenesis (Cummings and Bremner, 1994). Similar results were obtained when given as a continuous infusion. Only six out of eight subjects became oligozoospermic with 2 retaining normal sperm densities on infusion of 500µg LHRH agonist daily with 100mg TE every 2 weeks (Pavlou *et al.*, 1986). It appears that failure of spermatogenic suppression resulted from initial stimulation of gonadotrophin secretion ('flare') and subsequent 'escape', particularly of FSH (Behre *et al.*, 1992).

GnRH antagonists may increase the degree of gonadotrophic suppression and thus of spermatogenesis. Results from clinical studies have been more encouraging than with the agonists, with the administration of the prototype antagonist Nal-Glu with TE resulting in profound and rapid suppression of spermatogenesis (Pavlou *et al.*, 1987;

Tom *et al.*, 1992; Bagatell *et al.*, 1993). However, progress has been limited by their histamine-like side-effects on injection, their short duration of action, and the expense involved in their synthesis. This has however had the effect of stimulating research into biphasic administration protocols, with one drug regimen for the suppression phase followed by a lower dose maintenance phase. This was investigated using the GnRH antagonist cetrorelix in combination with 19-nortestosterone (200mg/ every 3 weeks) (Behre *et al.*, 2001). All men became azoospermic with the combined drug regimen, but when cetrorelix was discontinued and the androgen continued alone, spermatogenesis was restored. This may have resulted from inadequate androgen dosage, too prolonged a dosage interval, or because of poor FSH suppression from nortestosterone which is not aromatised. A second study involved administration of Nal-Glu with TE for 16 weeks and induced azoospermia in 10/15 men: subsequent TE-only 'maintenance' for 20 weeks sustained suppression in 13 out of 14 subjects, with only 1 showing escape (Swerdlhoff *et al.*, 1998). This dose of 100mg TE week alone is relatively ineffective in inducing azoospermia (Bebb *et al.*, 1996). Limiting GnRH treatment to the 'induction phase' may reduce costs as well as drug exposure. Orally active non-peptide GnRH antagonists have been described (Cho *et al.*, 1998) and may help alleviate the problems of drug cost and delivery, but no data relevant to this thesis are available. Recently a twice monthly injection of acyline (300mcg/kg) resulted in profound suppression of gonadotrophins and testosterone (Herbst *et al.*, 2004). However, its addition to a contraceptive regime to testosterone and levonorgestrel provided no additional suppression of gonadotrophins or testosterone over an 8 week treatment period (Matthiesson *et al.*, 2004). In this study numbers were small in this

treatment group (n=8) and the treatment period was only for 8 weeks, and further investigation of this approach with different contraceptive regimes may be promising.

1.10 ANDROGEN DELIVERY

1.10.1 Testosterone esters

Improving methods of androgen delivery remains one of the major hurdles in the development of male hormonal contraception. Testosterone is promptly degraded by the liver in first pass metabolism thus cannot be administered orally, with the exception of testosterone undecanoate. This ester is partially absorbed from the intestine in chylomicra thus avoiding first pass metabolism but it has a short duration of action with widely fluctuating plasma concentrations (Schümeyer *et al.*, 1983) with bioavailability of approximately 6% (Tauber *et al.*, 1986). Therefore, in addition to unpredictable pharmacokinetics with high inter and intra-individual variation, high oral doses are required. Oral TU has been investigated with oral CPA as a 'male pill' (Meriggiola *et al.*, 1996a) but these characteristics make it unsuitable for widespread use as a male contraceptive. Since triglycerides are rapidly absorbed by the lymphatics thus bypassing the liver, the pharmacokinetics of an oral testosterone-triglyceride conjugate (TTC) has been investigated and compared to oral TU in rabbits (Amory *et al.*, 2003). Maximum serum testosterone concentrations with TTC exceeded those of oral TU better absorption and improved bioavailability, with similar half-lives thereafter. However, the long-term effects of this oral testosterone preparation and potential suitability as part of a male contraceptive regime are unknown.

The most widely used preparations both in the above contraceptive studies and in hypogonadal replacement therapy involve esterification of 17 β -hydroxyl group with carboxylic acids. This increases the polarity of the molecule, making it more lipophilic and hydrophobic thereby slowing release from the injection site. Longer-acting testosterone preparations would improve the adverse pharmacokinetics of TE and similar preparations (Behre *et al.*, 1990; Behre and Nieschlag, 1992; Behre *et al.*, 1999) and necessitate a lower overall dosage for equivalent efficacy, as demonstrated with testosterone pellets (Handelsman *et al.*, 1992). Testosterone buciclate exemplifies this approach, with a terminal half-life of 29.5 days (Behre and Nieschlag, 1992) versus 4.5 days for TE (Behre *et al.*, 1990). In clinical studies, a single dose of 1200mg intramuscularly resulted in azoospermia in 3 out of 8 men while maintaining plasma testosterone levels in normal range (Behre *et al.*, 1995). However it is not currently available for further investigation. The undecanoate ester can also be administered intramuscularly with improved pharmacokinetics compared to TE, providing testosterone replacement for 6 to 8 weeks (Zhang *et al.*, 1998; Behre *et al.*, 1999; Nieschlag *et al.*, 1999). In a clinical study in China, 11 out of 12 volunteers became azoospermic with a dose of 500mg TU/4 weeks, whereas 12 out of 12 became azoospermic with the higher dose group (1000mg/4 weeks) (Zhang *et al.*, 1999). A recent efficacy study in China involving over 300 couples demonstrated a overall contraceptive efficacy of 94.8% with 500mg TU i.m. every 4 weeks (Gu *et al.*, 2003). Investigation of a similar TU preparation in a Caucasian population demonstrated a lower incidence of azoospermia when given alone at 1000mg/6 weeks (Kamischke *et al.*, 2001). However, in combination with the long-acting injectable progestin, norethisterone enanthate, 13 out of 14 men became azoospermic.

1.10.2 Testosterone Pellets

Subcutaneous implantation was among the earliest effective modalities for clinical application of testosterone. The first pellets were made from high pressure compression and included cholesterol. Currently available implants of fused crystalline testosterone are normally inserted surgically into the anterior abdominal wall under local anaesthesia. They have been in use since the 1950's in treating hypogonadal men and are fully biodegradable. They have near complete bioavailability with kinetics approximating zero-order release (Handelsman *et al.*, 1990) resulting in relatively stable serum concentrations. A dose of 800mg (4 x 200 mg pellets) provides physiological testosterone replacement for 4 to 6 months releasing approximately 6mg of testosterone per day (Handelsman, 1996) with good acceptability in hypogonadal men. In contraceptive studies, testosterone pellets alone at a dose of 1200mg achieved an equivalent degree of spermatogenic suppression to 200mg TE weekly, with fewer androgen related side effects (Handelsman *et al.*, 1992). Further dose-sparing resulted in decreased efficacy with only 4 out of 10 subjects becoming azoospermic with 800mg testosterone (Handelsman *et al.*, 1996). However, in combination with a single dose DMPA 300mg i.m., the extent of suppression was markedly increased with 9 out of 10 subjects becoming azoospermic. In a recent study in Edinburgh and Shanghai, the combination of 400mg testosterone pellets every 12 weeks with oral desogestrel resulted in azoospermia in all men while maintaining plasma testosterone concentrations in the physiological range throughout (Kinniburgh *et al.*, 2002). However, the pellets do require a minor surgical procedure for insertion and with an extrusion rate of 7%, a longer-acting depot injection seems the optimal choice at present. However their long duration of action and avoidance of initial

supraphysiological concentrations mean that the pellets remain a valuable prototype at present.

1.10.3 Topical testosterone

A more recent development is the transdermal delivery of testosterone, which can be administered by scrotal and non-scrotal routes. In comparison to female HRT when doses in the region of 50 to 100µg/day of estrogen are used much higher amounts of testosterone require to be delivered (3 to 10mg/day). Testoderm® (testosterone transdermal system, ALZA Corporation) patches in doses of 2.4 or 3.6mg, maintain testosterone concentrations in the adult physiological range for 24 hours when applied to the scrotum (Place *et al.*, 1990). They also elevate the DHT/T ratio to 0.5 (normal 0.1 to 0.2), reflecting the rich 5α-reductase activity in the scrotum. It is possible that this would have suboptimal effects in androgen target organs such as bone which are predominantly dependent on aromatisation of testosterone. Similarly, non-scrotal systems have been developed for use in hypogonadal men (Meikle *et al.*, 1992). Androderm® transdermal delivery systems (Teratech) at a dose of 2.5mg or 5mg maintain testosterone concentrations without altering the DHT/T ratio. However in one study, 72% of hypogonadal subjects elected to return to depot injections as a result of dermatological problems (Parker and Armitage, 1999). In a recent randomized parallel group study comparing transdermal testosterone 5mg/day with TE 200mg im/2 weeks in hypogonadal men, more physiological hormone levels were reported with reduced stimulation of erythropoiesis and gynaecomastia with the topical preparation. However, once again minor skin irritation was reported in 60% of patients (Dobs *et al.*, 1999), and these can be serious (Bennett, 1998). A further topical method of delivery involves the

application of testosterone and DHT gels. In studies using hydalcoholic DHT gels providing metered doses of 16mg/2.3g gel no skin irritation was reported (Wang *et al.*, 1998b). Applying metered doses of 16, 32 or 64mg provides testosterone replacement in the low, middle and high physiological range. As DHT is a more potent androgen than testosterone, less drug is required. The effect on the prostate is therefore reduced, as amplification of the effect of testosterone (by conversion to DHT) is avoided (Swerdloff and Wang, 1998; Ly *et al.*, 2001). Testosterone gels have also been shown to decrease bone resorption in hypogonadal men resulting in a significant increase in bone mineral density (Wang *et al.*, 2001).

In the 1980's, the concept of using a self-administered contraceptive regime was investigated with daily oral MPA 20mg with percutaneous testosterone 50/100mg per day and resulted in a significant reduction in sperm density with no reduction in libido (Soufir *et al.*, 1983). More recently, combination of oral desogestrel with transdermal testosterone (Andropatch®) resulted in less consistent suppression of spermatogenesis (66% with 300µg desogestrel) than with injectable esters. Marked skin irritation resulted in a significant number of men withdrawing from the study (Hair *et al.*, 2001). Similarly, only 2/11 volunteers reported no skin reaction using daily transdermal patches with oral levonorgestrel (125 or 250µg), and only 2/11 became azoospermic (Büchter *et al.*, 1999). Similar suboptimal suppression of spermatogenesis was also evident in combining transdermal T with Norplant II® and oral LNG (Gaw Gonzalo *et al.*, 2002) as previously discussed. In avoiding the necessity for repetitive injections and allowing self-administration, these methods of androgen replacement may prove very acceptable for use as part of a male hormonal contraceptive. However further work is

necessary in order to avoid the high incidence of local side-effects from patches and improve the degree of spermatogenic suppression.

1.10.4 7 α -Methyl-19-nortestosterone.

An alternate approach is the use of an androgen other than testosterone itself. Potential advantages might include increased potency and degrees of tissue selectivity allowing smaller quantities of drug to be administered with equipotent biological effects on some tissues such as the hypothalamus and pituitary gland, yet reduced activity in other tissues such as the prostate. Testosterone itself has a differential metabolism in different tissues: while testosterone itself is the active agent in skeletal muscle, aromatisation to oestradiol is important in peripheral tissues such as bone, liver, brain and adipose and 5 α -reduction to DHT is important in the prostate. One such androgen is 7 α -methyl-19-nortestosterone (MENT).

MENT is ten times more potent than testosterone in suppressing gonadotrophins and is resistant to 5 α -reduction (Kumar *et al.*, 1992) whilst it is aromatisable to an oestrogen (LaMorte *et al.*, 1994). It would therefore have the theoretical advantage of being relatively prostate sparing, which has in fact been demonstrated in castrate monkeys (Cummings *et al.*, 1998) and recently in humans (Anderson *et al.*, 2003). MENT does not bind to SHBG thus is rapidly cleared from the circulation. MENT acetate implants have been developed, and result in dose-dependent anti-gonadotrophic activity in healthy men (No   *et al.*, 1999) whilst maintaining mood and sexual behaviour in hypogonadal men to a similar degree as testosterone (Anderson *et al.*, 1999). In hypogonadal men, 2 implants releasing 400 μ g/day are required to maintain most

androgen-dependent functions other than bone mass (Anderson *et al.*, 2003). In combination with GnRH agonist, MENT has maintained azoospermia in non-human primates for 8 months (Sundaram *et al.*, 1987) and the contraceptive effect of sustained release MENT acetate implants has more recently been investigated in men (von Eckardstein *et al.*, 2003). Four implants (each releasing 400µg/day) were required to achieve azoospermia in 8/12 subjects, with oligozoospermia achieved in only 4/12 of the 2 implant group. Thus, they have an ability to suppress spermatogenesis and may confer significant advantages over other androgens with their tissue selectivity. Their potential role in male hormonal contraception needs further investigation and it would therefore be of value to examine their effects in combination with a second agent such as a gestogen.

1.11 CONTRACEPTIVE ACCEPTABILITY

For hormonal male contraception to become a reality, there need to be users as well as a method. Two recent surveys have addressed the attitudes of both men and women to this subject (Martin *et al.*, 1997; Glasier *et al.*, 2000). The lack of demand for more advanced male methods of contraception is often associated with the lack of research effort in this area. However, a third of couples worldwide rely on a male method of contraception suggesting that the lack of demand may be more perceived rather than real.

The first study aimed to investigate men's attitudes in different cultural settings to novel hormonal male methods of contraception (Martin *et al.*, 2000a). Approximately 450

men each from Edinburgh, Shanghai, Cape Town (giving samples of black, white and coloured men) and Hong Kong completed questionnaire-based interviews. The majority of men welcomed a new hormonal method with 40-83% saying that they would use a 'male pill' and felt it would have less of an impact than condoms on sexual desire and satisfaction. A pill was more acceptable than long-acting injections or implants except in Shanghai. Men in Hong Kong were least enthusiastic about novel male methods. This was the only centre that a male method (the condom) was the most commonly used approach, Despite this, Hong Kong men were the least likely to think that condoms were effective. On the contrary, in Edinburgh men were least likely to regard condoms as convenient although most likely to regard them as effective. The results indicate that acceptability was high among some groups, showing wide variability related to cultural background and current contraceptive usage. This study emphasizes that men wish to have greater involvement in family planning, although the reality of this will only be seen when further methods become available.

There is much speculation as to whether women would actually trust their partners to use a male pill. In a further study, the views of women were investigated to confirm whether this is reality or merely a commonly perceived myth. Over 1800 women attending Family Planning Clinics in Scotland, China and South Africa representing 3 main ethnic groups completed a questionnaire, with 65% expressing that the responsibility of contraception fell too much on women. Over 90% of women in Edinburgh and Cape Town felt that male contraception was a good idea allowing more equal sharing of responsibility with the Chinese only being slightly less positive (Hong Kong 71%, Shanghai 87%). Only 2% of women said that they would not trust their

partners to use such a method. 70-80% of Scottish and Chinese and 40% of black and coloured women in South Africa said that they would use it demonstrating the female acceptability of novel male methods.

There have only been two other similar studies, both from the USA and reporting a lower popularity for male contraception. In a survey carried out in the 1960's of 107 women attending family planning clinics or university students, the majority (72%) wanted to be in control of their own contraception with only 16% wanting men to take responsibility and 12 % wanting to share it (Bardwick, 1973). However, in a telephone survey of 1005 Americans (507 women) in the 1970's, 70% of men and women felt that men should have more responsibility for contraception: 45% of women thought that men would use a male pill (Henry J Kaiser Family Foundation, 1997). Although these results would imply a lower popularity compared to Scotland, the study population differed significantly: women were actually seeking advice and were also asked about their partners rather than men in general. Attitudes to partners would be different to men on the whole and may contribute to the higher acceptability evident in this survey (Glasier *et al.*, 2000). Despite the common belief that women would not want a male pill, these data suggest that not only would they want it but also that they would trust their partners to use it.

1.12 SUMMARY

Over the past decade we have seen significant progress in the quest to find a suitable male hormonal contraceptive. The available evidence suggests that both men and women would welcome this development (Martin *et al.*, 1990; Glasier *et al.*, 2000; Martin *et al.*, 2000a). Using testosterone enanthate alone resulted in sub-optimal efficacy in suppression of spermatogenesis. Recent efficacy studies with testosterone undecanoate alone, and results from combination regimes with progestogens are promising. Several problems remain with existing methods and the main aims for the future focus on improving methods of androgen delivery, reducing impacts on extra-testicular metabolism and increasing efficacy in suppression of spermatogenesis. In the following chapters of this thesis, the focus shall be on the gestogen/testosterone combined approach in the studies undertaken to investigate a potential efficacious, acceptable and safe male hormonal contraceptive.

CHAPTER 2

SUPPRESSION OF PITUITARY-TESTICULAR AXIS WITH ORAL ETONOGESTREL AND INTRAMUSCULAR TESTOSTERONE DECANOATE IN HEALTHY MEN

2.1 Introduction

The data discussed in this chapter are based on the Edinburgh cohort of a multi-centre study supported by Organon. I was responsible for the recruitment, management and data analysis of this cohort and will only report on this arm of the trial. The primary objective of this study was to investigate the efficacy of oral etonogestrel, the active metabolite of desogestrel, combined with the testosterone ester, testosterone decanoate in suppressing spermatogenesis over a treatment period of 1 year in healthy men. Further objectives included assessment of its safety and the pharmacokinetics of this regime.

As discussed in the preceding chapter, the concept of hormonal male contraception is based upon the administration of exogenous steroid to suppress pituitary gonadotrophins with the subsequent suppression of spermatogenesis (reviewed in (Anderson and Baird, 2002; Nieschlag *et al.*, 2003; Kamischke and Nieschlag, 2004; Wang and Swerdloff, 2004)). Earlier approaches involved the administration of androgen alone with effective suppression of spermatogenesis (World Health Organisation Task Force on Methods for the Regulation of Male Fertility, 1990, 1996). However, low rates of spermatogenesis were maintained in approximately one third of Caucasians with the resulting risk of pregnancy. Furthermore, supraphysiological

androgen levels resulted in significant side-effects on skin, haematopoiesis and serum lipoproteins (Wu *et al.*, 1996). The administration of a second agent such as a progestogen improves the degree of spermatogenic suppression as well as permitting a lowering of the dose of testosterone to approximately physiological replacement (Bebb *et al.*, 1996; Meriggiola *et al.*, 1996b). Several gestogens have been investigated including levonorgestrel (Bebb *et al.*, 1996), cyproterone acetate (Meriggiola *et al.*, 1996b; Meriggiola *et al.*, 1998), medroxyprogesterone acetate (Knuth *et al.*, 1989; World Health Organisation Task Force on Methods for the Regulation of Male Fertility, 1993; Handelsman *et al.*, 1996; Turner *et al.*, 2003), norethisterone (Kamischke *et al.*, 2001) and desogestrel (Wu *et al.*, 1999; Anawalt *et al.*, 2000; Kinniburgh *et al.*, 2002). Results with desogestrel have been promising, with near universal suppression of spermatogenesis. It has been previously demonstrated that in combination with testosterone, desogestrel can suppress spermatogenesis without significant side-effect (Wu *et al.*, 1999; Martin *et al.*, 2000b; Kinniburgh *et al.*, 2002). The optimal dose has been demonstrated to be 300 micrograms (Bellis *et al.*, 1996) with no increase in efficacy and speed of onset of suppression from a further increase in dose to 450 micrograms. There is no difference in the comparative pharmacokinetics between desogestrel and etonogestrel in women, therefore a similar dose of 300 micrograms for etonogestrel (Hasenack *et al.*, 1986) was used in this study.

The lack of availability of a convenient long-acting injectable testosterone preparation has been a major obstacle to the development of hormonal male contraception. Previous studies with testosterone enanthate relied on weekly injection intervals. Not only may this be unacceptable to volunteers, but supraphysiological testosterone peaks

were observed. Testosterone Decanoate, is another long-acting ester, hydrolysed to testosterone in the circulation and is the major component in Sustanon ® which has been used in the treatment of hypogonadal men for several years. Preliminary data demonstrated that 400mg administered every 4 weeks (with etonogestrel implants) resulted in good spermatogenic suppression and maintenance of physiological testosterone concentrations (Anderson *et al.*, 2002c). The similar ester testosterone undecanoate, at a dose of 1000mg maintains testosterone levels within the physiological range in hypogonadal men for 6 to 8 weeks (Behre *et al.*, 1999; Nieschlag *et al.*, 1999). A dose of 400mg every 6 weeks may lead to infra-physiological concentrations. However we hypothesised that because etonogestrel lowers SHBG, bio-available testosterone concentrations may maintain normal androgenic function and therefore this dose was further explored in treatment group II.

2.2 Subjects and methods

Subjects and treatment medication

Twenty healthy men were recruited from the general population (mean age 30 years, range 21-44 years). Inclusion criteria included age (18-45), no significant past medical history, Body Mass Index between 18-30 kg/m³, normal pre-treatment FSH, LH and testosterone concentrations, 2 normal semen analyses according to WHO criteria (WHO, 1999) at least 2 weeks apart, and a normal physical and andrological (prostate and testes) examination. Subjects were also willing to provide written informed consent and the study had ethical approval from Lothian Reproductive Medicine Ethical Review Committee. The study was performed according to GCP guidelines (MRC, 1998).

Open randomisation was done centrally by means of an Interactive Voice Response System, assigning subjects to one of the 2 treatment groups.

Oral desogestrel (NV Organon, Oss, The Netherlands) was taken at a dose of 300 micrograms (2 x 150 microgram tablets) daily by all subjects. Testosterone Decanoate (NV Organon, Oss, The Netherlands) 200mg/ml in vials with castor oil was administered by intramuscular injection at a dose of 400mg every 4 (group I) or 6 (group II) weeks. Subjects were instructed to take 2 etonogestrel tablets in the morning and compliance checked by subjects completing drug diaries and checking the packets every month. Any concomitant medication was noted throughout the study.

Treatment protocol

Subjects were randomized to one of 2 treatment groups for a period of 48 weeks:-

Group I: 300 µg etonogestrel and 400mg TD 4 weekly (see Table 2.1)

Group II: 300 µg etonogestrel and 400mg TD 6 weekly (see Table 2.2)

During screening subjects had a medical history and examination performed. Testicular volume was assessed by Prader orchidometry and prostate volume by trans-rectal ultrasonography (prior to blood sampling). Bloods were taken for full blood count (FBC), clinical chemistry, lipids, gonadotrophins, Prostate Specific Antigen (PSA) and glucose. Two semen samples were submitted following a period of abstinence of 2 to 7 days. During treatment and recovery subjects attended to submit a semen sample every 28 ± 3 days. Throughout the study bloods were drawn at regular intervals to assess gonadotrophins, testosterone, DHT and SHBG. Peak testosterone concentrations were assessed in extra visits at weeks 1, 2, 17 and 18 in Group I and at weeks 1, 2, 19 and 20

Pre-treatment	Treatment																	
Week	-4	0	1	2	4	8	12	16	17	18	20	24	28	32	36	40	44	48
Physical exam	✓					✓						✓			✓			✓
Routine labs ¹ & PSA	✓					✓						✓			✓			✓
ENG/TD					✓	✓						✓			✓			✓
T/DHT/SHBG	✓		✓	✓	✓	✓		✓	✓		✓	✓			✓			✓
FSH/LH	✓				✓	✓		✓				✓			✓			✓
Semen sample	✓				✓	✓	✓	✓			✓	✓	✓	✓	✓	✓	✓	✓
Testes & prostate vol	✓					✓						✓			✓			✓
DISF-SR/ mood	✓					✓						✓			✓			✓
Drug accountability		✓			✓	✓	✓	✓	✓		✓	✓	✓	✓	✓	✓	✓	✓
TD injection		✓			✓	✓	✓	✓			✓	✓	✓	✓	✓	✓	✓	

Table 2.1: Flow chart of subject assessments in (pre)treatment phase of Group I receiving TD 400mg i.m. every 4 weeks.
¹Includes haematology, biochemistry, liver function tests, fasting and fasting lipids. PSA was drawn before prostate examination

Week	Pre-treatment				Treatment												
	-4	0	1	2	4	6	8	12	16	18	19	20	24	30	36	42	48
Physical exam	✓						✓						✓		✓		✓
Routine labs ¹ & PSA	✓						✓						✓		✓		✓
ENG/TD					✓	✓							✓		✓		✓
T/DHT/SHBG	✓		✓	✓	✓	✓		✓		✓	✓	✓	✓		✓		✓
FSH/LH	✓				✓	✓	✓	✓		✓			✓		✓		✓
Semen sample	✓			✓			✓	✓	✓			✓	✓	✓	✓	✓	✓
Testes & prostate volume.	✓						✓					✓	✓		✓		✓
DISF-SR/mood	✓						✓						✓		✓		✓
Drug accountability		✓			✓		✓	✓	✓		✓	✓	✓	✓	✓	✓	✓
TD injection		✓				✓		✓		✓			✓	✓	✓	✓	

Table 2.2: Flow chart of subject assessments in (pre)treatment phase of Group II receiving TD 400mg i.m. every 6 weeks.
¹Includes haematology, biochemistry, liver function tests, fasting and fasting lipids. PSA was drawn before prostate examination

in Group II. Subjects were examined at weeks 8, 24, 36, 48 and at final visits. Sexual functioning and mood were assessed by means of Derogatis Interviews (DISF-SR) (Derogatis, 1997). Throughout the study any adverse events were noted at each visit. During recovery, subjects attended at 4 weekly periods for a minimum of 16 weeks up to 24 weeks until semen concentrations returned to normal by WHO criteria (WHO, 1999). Subjects with semen analysis below normal WHO criteria were followed up beyond this period during a period of extended follow-up until normal values were attained.

Hormone assays

Blood samples were obtained in fasting subjects (for glucose and lipids), and plasma separated by centrifugation at 4000g for 15 minutes and stored at -20C until hormone assay by a central laboratory (NV Organon). SHBG, FSH and LH were all determined by time-resolved immunofluorometric Delfia assays (Perkin-Elmer, Wallac). Testosterone concentrations were determined using a gas chromatographic assay with mass spectrometric detection after solid phase extraction, derivatisation and liquid-liquid extraction with n-hexane. The limit of quantification (assay sensitivity) for testosterone was 0.34nmol/L, FSH (0.248U/L) and LH (0.52U/L). Intra and interassay coefficients of variation were 1.9-6.7%(FSH), 2.6-4.9%(LH) and 8.3-11.7%(testosterone) respectively. Assay sensitivity for SHBG was 6.25nmol/L with intra and interassay coefficients of variation of 3.2-5.0%. Etonogestrel was measured by an in-house RIA (NV Organon) with an assay sensitivity of 30pg/mL. HDL-C was measured after precipitation with dextran sulphate-magnesium, and total cholesterol and triglycerides measured enzymatically (Wood *et al.*, 1987). LDL-C was measured

indirectly (Friedewald *et al.*, 1972). Haemoglobin, renal and liver function tests were determined by routine autoanalyser.

Semen analysis

Semen samples were obtained following masturbation into pre-weighed pots after an abstinence period of between 2 and 7 days and protected from extremes of temperature on transportation to the laboratory. The andrology laboratory was subject to both internal and external Quality Control assessment. Abstinence and time of ejaculation was noted for all semen samples. At all assessments semen analysis was carried out by WHO criteria (WHO, 1999) examining ejaculate consistency, volume, sperm concentration, morphology and motility. Motility was examined within 60 minutes of ejaculation at a temperature of 37°C. Normal reference values were: volume > 2ml, pH > 7.2, sperm concentration > 20M/ml, total sperm number > 40 M/ejaculate, vitality > 50%, white blood cells < 1M/ml. According to our local laboratory, normal motility was considered to be > 27% (grade a (rapid progressive) + b (slow progressive)) or > 36% (a + b + c (non-progressive)) and normal morphology > 15 %. Azoospermia was confirmed following microscopy by centrifugation of the whole semen sample. Centrifugation was performed at 3660 rpm (revolutions per minute) for 15 minutes and a sample classified as azoospermic after a complete and systematic examination of the re-suspended precipitate.

Behavioural assessment

Sexual functioning and mood were assessed by self-completed questionnaires every 3 to 4 months. The Derogatis Interview for Sexual Function self-report (DISF-SR) is a set

of gender-related outcome measures designed to measure overall quality of sexual functioning (Derogatis, 1997). The 5 primary domains assessed were:

- I: Sexual cognition and fantasy
- II: Sexual arousal
- III: Sexual behaviour and experience
- IV: Orgasm
- V: Sexual drive and relationship

The DISF overall total score was calculated for each individual throughout the study. Mood was also assessed by means of an unvalidated mood questionnaire consisting of 13 mood related questions (Scale of 1 “all of the time” to 6 “none of the time”) allowing an overall score to be calculated

Prostate Volume

Prostate volume was determined by trans-rectal ultrasonography using an endocavity transducer (PVM 740RT) at a frequency of 7MHz (viewing angle 140 degrees; Toshiba Diagnostic Ultrasound System model SSA-340A). Maximal height and width measurements were determined with axial scans at the largest appearing mid-gland level. Length was determined as the distance from prostate apex to the bladder base on midline sagittal scan. Volume was then calculated in cm^3 using the prolate ellipse volume calculation ($\pi/6$ (width x height x length)). These were carried out by a single operator for consistency and accuracy of comparison throughout the study. Although less accurate than planimetry, this method was a rapid volume measurement technique, did not require additional equipment and could be simply carried out by a sole operator.

It has also been reported to be more accurate than ellipsoid software packages (Littrup *et al.*, 1991).

Body Composition

Bio-electrical impedance was determined as described (Davies and Preece, 1988; Gregory *et al.*, 1991) using the Holtain Body Composition Analyser (Holtain Limited, Dyfed, Wales). Measurements were taken within 30 minutes of voiding and with no previous alcohol ingestion for at least 48 hours. Impedance plethysmograph electrodes were placed on the back of the right wrist joint and front of the right ankle with the subject in the supine position and a 50 KHz current applied. An excitation current is introduced at the distal electrodes and the voltage drop detected by the proximal electrodes. This voltage drop gives the impedance of the body (according to Ohm's law, Impedance = Voltage/Current). The volume of a conducting medium i.e. Total Body Water = Conductor length (height²) / Impedance. Together with the subjects weight and height, a programmed 'Psion Organiser' was used to determine Total Body Water (TBW), Fat Free Mass (FFM), Fat Mass (FM) and % Body fat for each subject at screening, 8, 24, 36 and 48 weeks and at the Final visit.

Statistical Analysis

Results are presented as mean \pm SEM throughout. Whilst sperm concentrations are not normally distributed, these data are conventionally represented as mean \pm SEM. This was only used as a convenient representation of the data and did not form the basis of statistical analysis. Baseline sperm concentrations are also presented as median \pm IQR.

Data was transformed (log transformations for hormones and cube root for sperm concentrations) to give normal distributions of data prior to analysis. ANOVA was performed for hormone data for repeated measures. Paired t-tests were performed to investigate at what time points significant effects were evident in both groups I and II. Categorical data (behavioural) was examined by non-parametric testing. As the data in this chapter were based on a single cohort of a multicentre study, a power calculation was not performed.

2.3 Results

Pre-treatment values, adverse events and withdrawals

All pre-treatment values are presented in table 2.3. The mean age of subjects was 30 (range: 21 – 44). There were no statistically significant differences in mean age, BMI, sperm concentrations, mean testosterone and FSH concentrations. However, group II had a significantly higher LH concentration than group I: 5.11 ± 0.44 vs $3.38 \pm 0.28/4$ (group II vs I, $p=0.002$).

Ensuring drug compliance is essential in investigating an orally self-administered agent. Compliance with medication was checked by subject recording tablet taken in diaries on a daily basis and tablet blisters were returned on a monthly basis for inspection. Percentage drug intake compliance (calculated by total number of drugs taken/number of scheduled intakes during the actual treatment period) was calculated for each subject. Compliance was 97.7% and 99.1% in groups I and II respectively. Only two subjects did not take medication for more than 3 consecutive days.

Three subjects from group II withdrew before completing the treatment period (all from group II). The first subject discontinued treatment at 16 weeks due to mood disturbance. The second subject discontinued at 12 weeks again due to mood disturbance and significant weight gain (9kg). The third subject discontinued after 36 completed weeks of treatment due to a change in work and personal circumstances. Other adverse events in group II included reduced libido (2), lethargy (1), and mood

Table 2.3: Pre-treatment values of the two treatment groups receiving oral etonogestrel and intramuscular testosterone decanoate (TD).

Pretreatment value	Group I 400mg/4wk (n=10)	Group II 400mg/6week (n=10)
Age (yr)	28.2±1.2	31.9±1.95
Body Mass Index (kg/m ²)	24.2±0.96	25.7±0.65
Sperm concentration (x10 ⁶)	116.7±16.6	99.9±20.8
Sperm concentration (x10 ⁶)*	104.5±105	54.5±145
LH (IU)	3.38±0.28	5.11±0.44**
FSH (IU)	4.35±0.57	4.80±0.63
Testosterone (nmol/litre)	23.3±2.0	23.5±1.65

Group I: 300µg etonogestrel p.o. daily and 400mg TD i.m. 4 weekly
Group II: 300µg etonogestrel p.o.daily and 400mg TD i.m. 6 weekly
Data presented as mean±SEM. *Data presented as median±IQR
**p=0.002 Group II vs I.

disturbance (3). In group I adverse events included reduced libido (1), and increased sweating (1).

Sperm concentrations

A profound fall in sperm concentration was observed in both groups receiving oral desogestrel with testosterone decanoate (Figure 2.1a). This reached statistical significance at 4 weeks in both groups. Mean sperm density fell from 116.7 ± 16.6 to 19.3 ± 11.4 ($p=0.0004$) in group I and from 96.38 ± 23.25 to 23.91 ± 4.72 ($p=0.016$) in group II by 4 weeks of treatment. Suppression of spermatogenesis was to a lesser extent in group II than in group I. By week 24, azoospermia was achieved in 8/10 subjects in group I and 4/8 subjects in group II. At the same time, sperm concentrations had fallen in all 10 subjects in group I to less than 1M/ml ($9/10 < 0.1\text{M/ml}$) and in 6/8 subjects (all $< 0.1\text{M/ml}$) in group II (Figure 2.1b). In addition, the 2 subjects who dropped out in the early treatment phase in group II attained concentrations of less than 1M/ml by week 8 of treatment and including these, 8/10 subjects would have achieved this threshold in this group within the first 24 weeks of treatment. In the latter half of the treatment period, both ongoing suppression and 'rebound' from suppression of spermatogenesis was observed in both groups. In group I, by week 48 all 10 subjects were azoospermic. However during that period, 'rebound' from suppression was observed in 2 of these subjects, albeit to levels of $< 0.1\text{M/ml}$. In group II, 6 out of 8 subjects were azoospermic ($7/8 < 1\text{M/ml}$) by week 48. Of the 2 subjects who were not azoospermic in group II, 'rebound' from azoospermia (at week 36) was observed in one

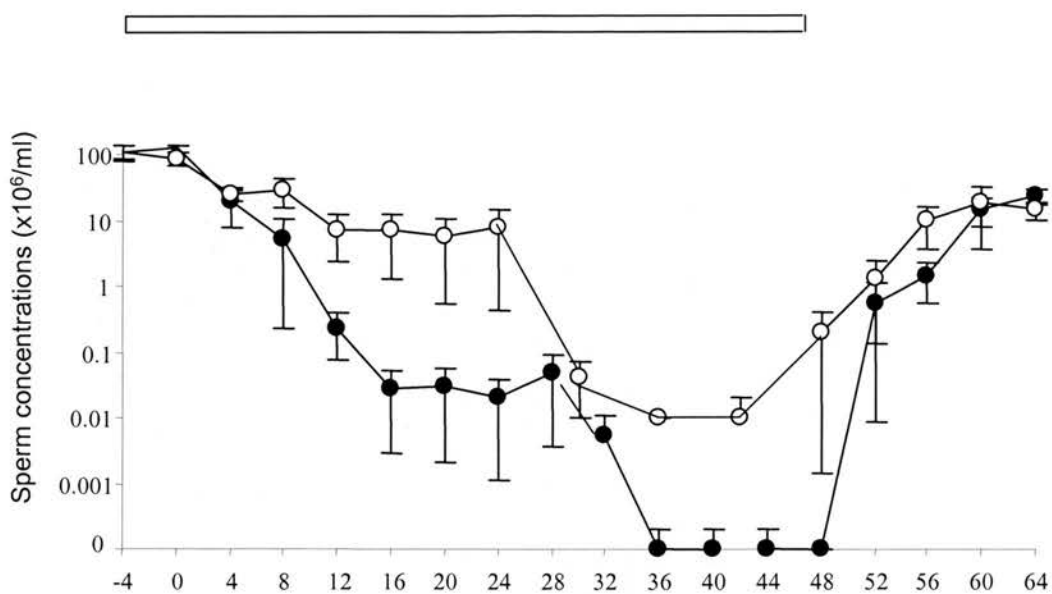


Figure 2.1a: Sperm concentrations during oral etonogestrel and 400mg TD i.m. every 4 weeks ●, or 6 weeks ○. Treatment period indicated by double bars. Data presented as mean+/-sem. Note logarithmic scale on ordinate.

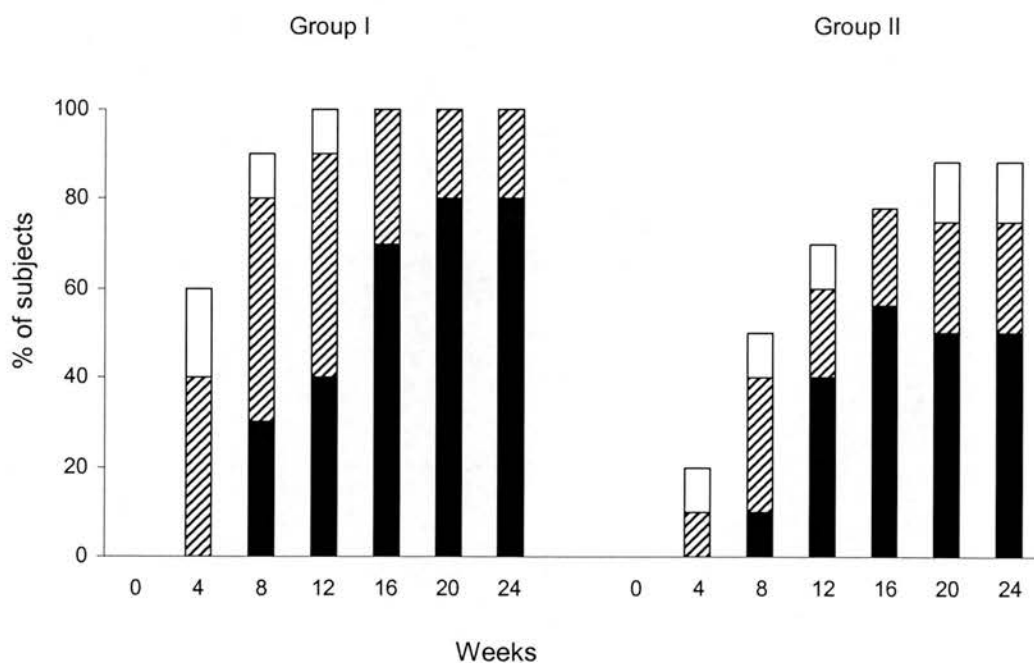


Figure 2.1b: Percentages of men achieving azoospermia ■, sperm concentrations of 1×10^6 /ml or less ▨, and 3×10^6 /ml □ or less at each time point during oral etonogestrel and TD treatment.

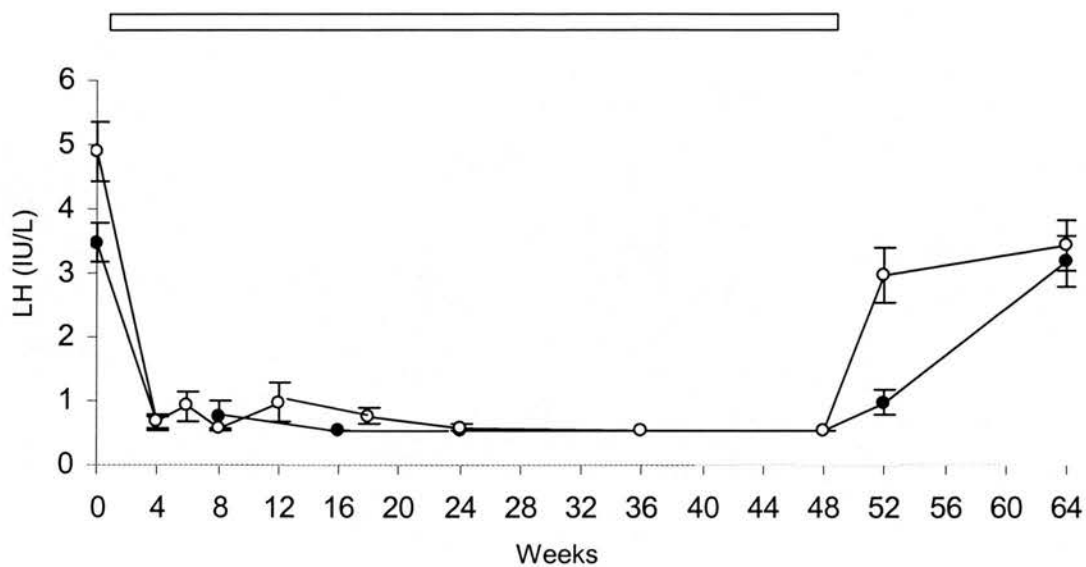
and the other unfortunately did not produce a sample at week 48, although had reached the suppression threshold of $<0.1\text{M/ml}$ at 36 weeks.

Although suppressed to a lesser extent in group II, recovery of spermatogenesis was slower with a median time to recover at least one sperm concentrations greater than 20M/ml of 126 days, compared to 112 days in group I. At week 16 of follow-up, mean (and median) sperm concentrations were 22.4 ± 5.4 (27.0) and 14.0 ± 4.0 (11.5) in groups I and II respectively. By week 24 of follow-up, 8/10 subjects in group I and 5/8 in group II had recovered to normal levels (at least 1 sample). The remaining 5 subjects (2 in group I and 3 in group II) entered a period of prolonged follow-up. In group I, one subject recovered spermatogenesis at week 40-44 and the other at week 56. In group II, recovery was earlier, one subject at week 28 and the remaining two at week 40.

Gonadotrophins

A profound suppression of gonadotrophins was observed in both treatment groups. This was statistically significant for both gonadotrophins in both groups by week 4 of treatment ($p < 0.001$ for both). This maintenance of this suppression was less consistent in group II (400mg/6 weekly) (Figure 2.2). In group I, LH was suppressed to undetectable levels in all subjects by week 16 and remained undetectable for the duration of the treatment period. Similarly FSH was suppressed to undetectable levels in all subjects by week 16 and remained undetectable for the majority of samples thereafter, with only marginally detectable levels in 3 samples over the entire treatment period. In group II, although LH levels are eventually undetectable at week 36: they fluctuated throughout the treatment period and did not remain fully suppressed after

(a)



(b)

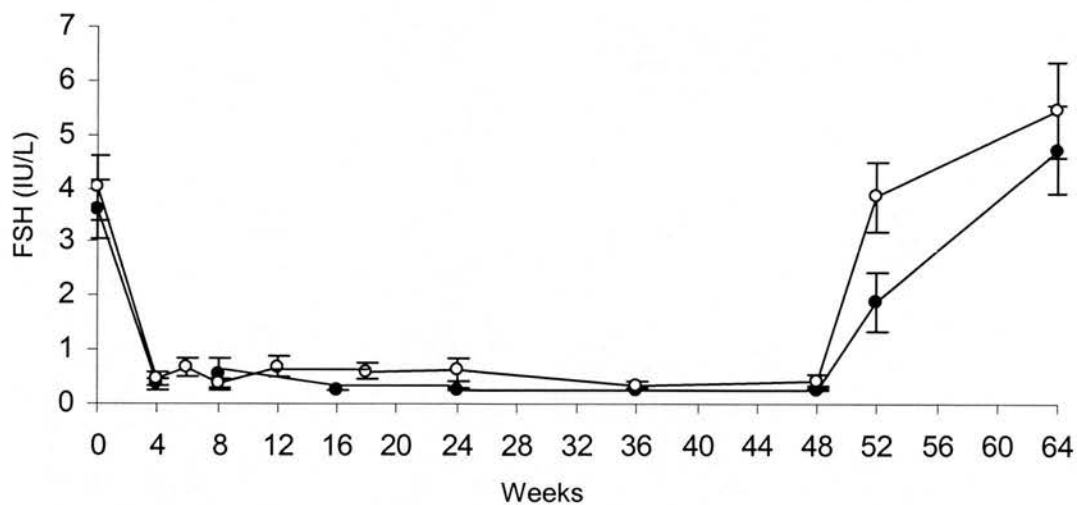


Figure 2.2 (a) LH and (b) FSH concentrations during oral etonogestrel and TD treatment. The duration of treatment is indicated by the double bars.
● Group I TD every 4 wks. ○ Group II TD every 6 wks. Data presented as mean \pm sem.

Table 2.4: Haematological parameters, lipids and SHBG during treatment and at final visit with oral etonogestrel and i.m. TD.

Group I (400mg/4 weekly), n=10					Group II (400mg/6 weekly), n=8				
Week	0	24	48	final	0	24	48	final	final
Hb (g/L)	153.8±2.3	155.9±2.0	163.3±2.3 ^a	158.1±1.6 ^a	149.5±3.5	145.9±2.2	154.9±3.0	151.1±2.3	
Hct	0.45±0.01	0.45±0.01	0.48±0.01 ^b	0.46±0.01	0.44±0.01	0.43±0.01	0.45±0.01	0.45±0.01	
Cholesterol (mmol/L)	5.4±0.5	4.7±0.2 ^c	4.9±0.3 ^c	5.4±0.4	5.4±0.5	4.9±0.4	5.3±0.4	5.4±0.4	
HDL-C (mmol/L)	1.2±0.1	0.9±0.1 ^d	1.0±0.1 ^d	1.2±0.1	1.3±0.1	1.1±0.1 ^e	1.0±0.1 ^e	1.3±0.1	
LDL-C (mmol/L)	3.6±0.4	3.1±0.2	3.3±0.3	3.3±0.3	3.5±0.5	3.3±0.4	3.5±0.5	3.5±0.4	
TG (mmom/L)	1.3±0.1	1.4±0.2	1.4±0.3	2.0±0.3 ^f	1.3±0.3	1.2±0.2	1.6±0.3	1.3±0.3	
SHBG (nmol/L)	39.4±3.7	22.3±2.2 ^g	24.9±2.7 ^g	33.6±2.6	37.0±4.9	22.6±2.2 ^h	22.9±2.9 ^h	33.2±5.0	

^a Hb increased significantly in group I at wk48 (p=0.0004) and recovery (p=0.03). ^b Significant increase in Hct in group I (p<0.001) at wk 48. ^c Significant decline in total cholesterol at wk 24 (p=0.048). ^d Significant decline in HDL-C in group I (p<0.001) and ^e group II (p<0.01) from wk 24, returning to baseline thereafter. ^f Significant increase in triglycerides (TG) in group I after recovery: 2 individuals had exceptionally high concentrations and may account for this. ^g Significant decline in SHBG in group I (p<0.001) and ^h group II (p<0.01) from week 24 returning to baseline thereafter. All data presented as mean±sem and p-values versus week 0.

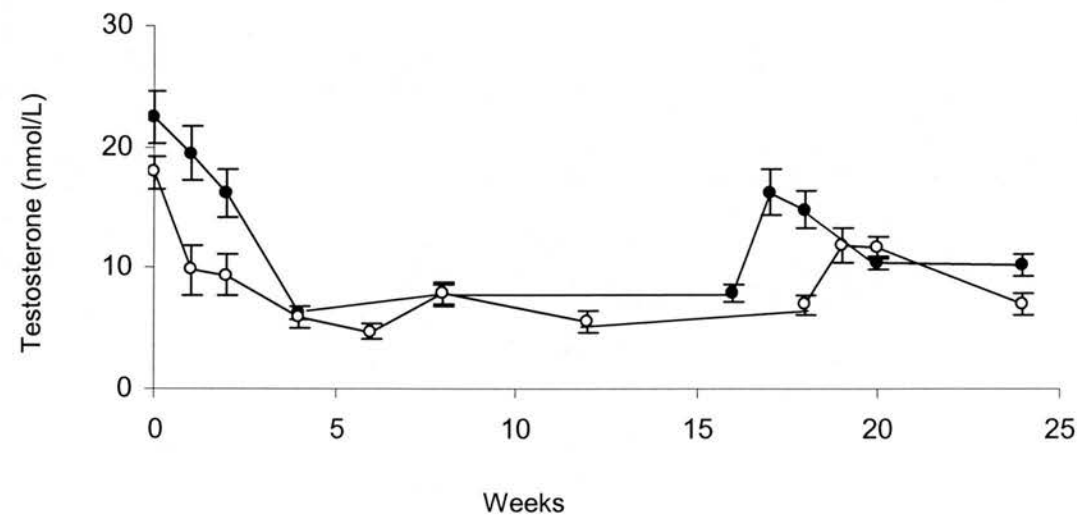
week 36. Similarly for FSH in group II, although significantly decreased by week 4 ($p=0.0005$), mean levels remained above levels of detection for the duration of treatment. This is also reflected in the recovery of both LH and FSH which was quicker in group II: mean levels at week 4 of recovery were 2.97 ± 0.42 versus 0.98 ± 0.19 ($p=0.001$) and 3.84 ± 0.67 versus 1.90 ± 0.55 ($p=0.03$) for LH and FSH in groups I and II respectively.

Other reproductive hormones

SHBG levels decreased rapidly during etonogestrel and testosterone treatment by approximately 40% in both groups (Table 2.4). This decline reached statistical significance by week 1 of treatment in both groups: concentrations fell from 39.4 ± 3.7 to 30.4 ± 2.9 ($p=0.002$) and from 37.0 ± 4.9 to 27.8 ± 3.3 ($p=0.04$) in groups I and II respectively. Concentrations returned to baseline levels after follow-up.

Mean testosterone concentrations fell early in the treatment period such that mean levels were sub-physiological by week 4 in both groups (Figure 2.3a). Mean trough levels in group I were initially below normal range (6.28 ± 0.47 at week 4) although demonstrated a cumulative effect reaching the physiological range by week 20 and remaining in normal range for the rest of the treatment period. Similarly in group II, mean trough testosterone concentrations were low (4.71 ± 0.63 at 6 weeks) and remained in the sub-physiological range throughout the treatment period (Figure 2.3b). Peak testosterone concentrations were assessed after the first and fourth TD injections (weeks 1, 2, 17 and 18 in group I and 1, 2, 19 and 20 in group II) and were within the normal range (Figure 2.3a). By week 4 of follow up, mean testosterone concentrations had returned to normal

(a)



(b)

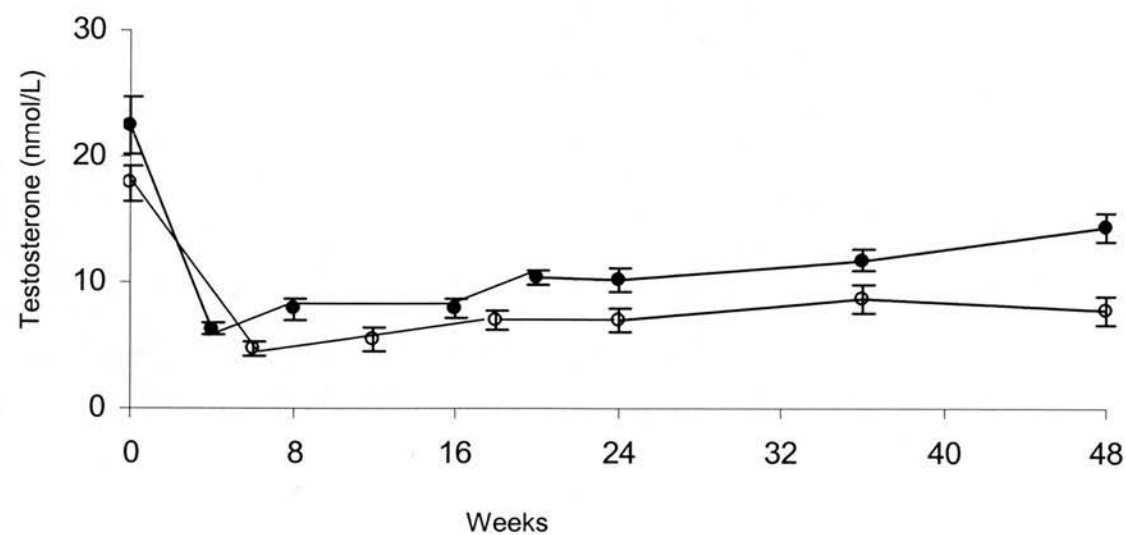
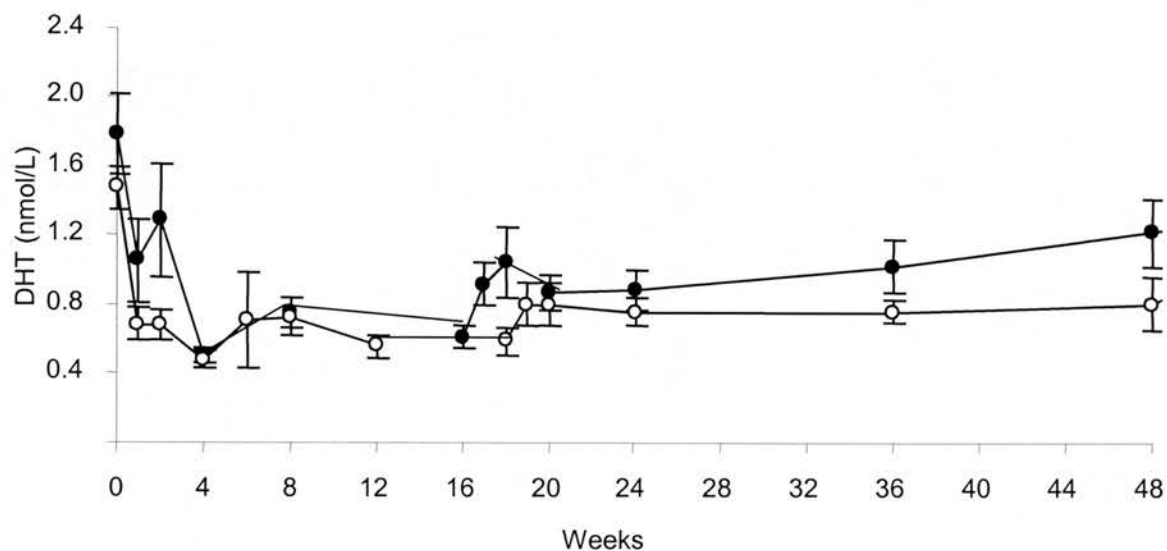


Figure 2.3 (a) Mean testosterone during weeks 0-25 and (b) mean trough testosterone concentrations during oral etonogestrel and im TD treatment. Data presented as mean \pm sem.

- Group I (400mg TD/4wk).
- Group II (400mg/6wk).

(a)



(b)

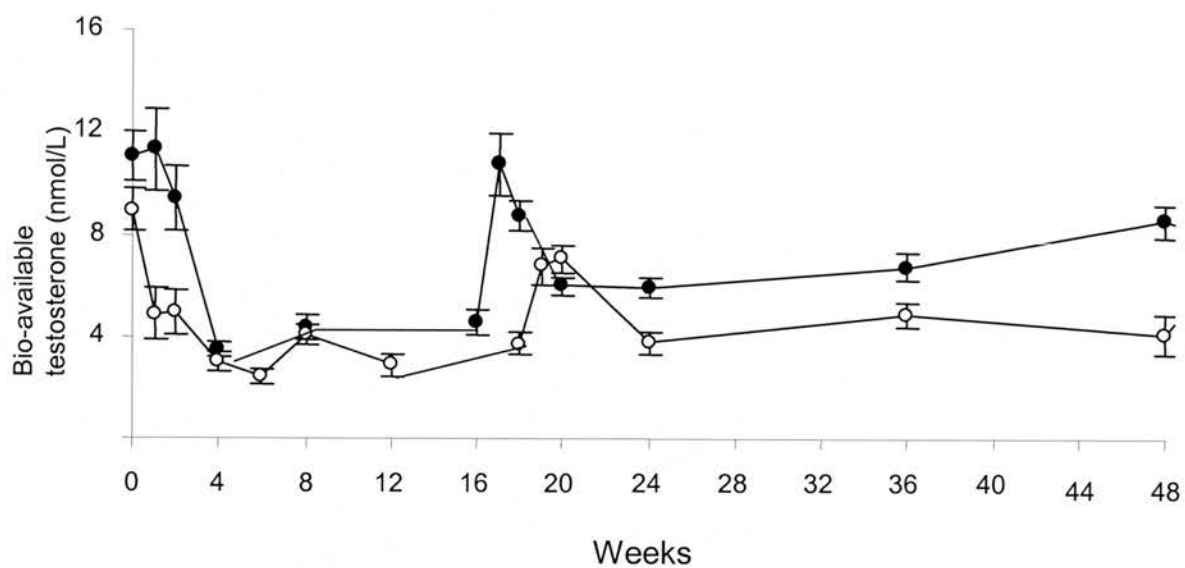


Figure 2.4 (a) DHT (nmol/L) and (b) Bio-available testosterone (nmol/L) during oral etonogestrel and im TD treatment. ● Group I (400mg TD 4/wk) and ○ Group II (400mg TD/6 wk). Data presented as mean \pm sem.

range. Bio-available testosterone and DHT (Figure 2.4a & 2.4b) showed a similar pattern of fluctuation during the study.

Etonogestrel

Mean etonogestrel concentrations were variable throughout. Mean concentrations (pg/ml) in group I were 537.5 ± 120.4 and 1306.9 ± 410.1 and in group II 1952.0 ± 334.7 and 1953.5 ± 380.5 at weeks 24 and 48 respectively. It was intended that these were representative of trough levels; however blood sampling may not always have been taken prior to drug ingestion and may account for the variability in these data.

Metabolic

There were significant increases during treatment in both haemoglobin and haematocrit in Group I. Haemoglobin increased from 153.8 ± 2.3 pre-treatment to 163.3 ± 2.3 ($p=0.004$) and haematocrit from 0.45 ± 0.01 to 0.48 ± 0.01 ($p=0.0008$) at week 48 of treatment, returning to baseline after follow-up. Although statistically non-significant, a small increase in haemoglobin was observed in group II with no significant increase in haematocrit (Table 2.4).

Both groups demonstrated a fall in both mean total cholesterol and HDL-C concentrations (Table 2.4). Total cholesterol concentrations fell significantly in group I by week 24 returning to baseline levels in follow-up. The decline in mean HDL-C concentrations was significant in both groups. In group I, HDL-C fell from 1.22 ± 0.08 to 0.97 ± 0.05 ($p<0.0001$) and in group II from 1.32 ± 0.09 to 1.0 ± 0.06 ($p=0.015$) at pre-

Table 2.5: Clinical & behavioural parameters: weight, body composition, prostate data and overall DISF-SR scores following oral etonogestrel and TD treatment and during follow-up

Week	Group I (400mg/4 weekly), n=10				Group II (400mg/6 weekly), n=8			
	0	24	48	final	0	24	48	final
Weight (kg)	79.5±3.6	80.9±3.3	83.2±3.8 ^a	82.6±3.9	86.5±3.2	85.6±3.2	87.5±3.4	88.6±3.7
FFM (kg)	60.3±1.5	61.7±1.7	65.7±1.3 ^b	64.4±1.3	63.5±1.9	63.0±3.0	63.3±4.3	67.7±3.0 ^c
FM (kg)	19.1±2.6	19.2±2.3	17.5±3.0	19.1±3.1	21.4±2.5	21.6±2.8	23.9±4.2	22.0±3.1
Prostate Volume (cm ³)	18.0±1.3	19.7±1.9	18.9±1.7	19.2±1.6	18.6±1.2	19.7±1.8	19.3±2.6	20.6±1.2
PSA (µg/L)	0.8±0.1	0.8±0.1	1.0±0.1 ^c	1.1±0.2	0.8±0.1	0.9±0.1	0.8±0.1	1.0±0.3
Total DISF-SR score	103.4±3.4	101.9±3.6	108.2±3.3	107.4±4.2	101.8±4.3	103.6±4.1	95.9±4.6	97.9±4.6

^a Significant increase in weight in group I at week 48 (p<0.0001). ^b Significant increase in fat free mass (FFM) in group I (p=0.0002) at wk 48 and ^c increase in group II (p=0.035) at final visit. ^c Significant increase in PSA at week 48 (p=0.03). FM = fat mass. Data presented as mean±sem, and all p-values versus week 0.

treatment and weeks 48 respectively. Both total cholesterol and HDL-C returned to normal levels during follow-up.

Physical and Behavioural

Body weight increased in both groups during treatment. This was greater and significant in group I, with an increase by approximately 5% by week 48 ($p < 0.0001$) returning to baseline levels thereafter (Table 2.5). In group II, the increase by the end of treatment was less (approximately 1%) and remained elevated through the follow-up period. A similar pattern was observed with fat free mass, increasing in both groups and reaching statistical significance by week 48 in group I and at the end of follow-up in group II. Fat mass did show any significant change during oral etonogestrel and TD treatment or follow-up. Prostate volumes did not change significantly during the study although there was a small but significant increase in PSA at week 48 in group I (this may be attributed to one subject who had a urinary tract infection at that time) (Table 2.5).

Sexual behaviour assessed by DISF-SR did not demonstrate and change on total scores (Table 2.5), or the sub-categories previously described.

2.4 Discussion

The combination of oral etonogestrel and injectable testosterone decanoate results in a profound suppression of spermatogenesis. By 24 weeks of treatment azoospermia was achieved in 50-80% of subjects and severe oligozoospermia (to $<1\text{M/ml}$) in 75-90%. Ongoing suppression of spermatogenesis increased this efficacy in suppression to azoospermia in 75-100% and to $<1\text{M/ml}$ in 88-100% of subjects at week 48 of treatment. This is an important finding as few male hormonal contraceptive studies to date (Kinniburgh *et al.*, 2002; Turner *et al.*, 2003) have investigated progestogen/androgen combined regimes for the time period of 1 year. These results are comparable to other studies, mostly of shorter duration, investigating oral desogestrel in combination with testosterone. In these studies azoospermia was achieved in 57-100% and oligozoospermia in 67-100% of subjects (Wu *et al.*, 1999; Anawalt *et al.*, 2000; Anderson *et al.*, 2002b; Kinniburgh *et al.*, 2002). Similar metabolic effects to other studies with a progestogen/androgen combination are observed in this study with a decline in HDL-C and weight gain (Bebb *et al.*, 1996; Wu *et al.*, 1999; Anawalt *et al.*, 2000).

It is clear on comparing the two groups that there is a difference in the pattern of spermatogenic suppression with lesser suppression in group II, receiving the lower dose of 400mg TD every 6 weeks. Underlying mechanisms that may contribute to this include the lesser suppression of gonadotrophins, and the lower dosage interval of TD in group II with consequent sub-physiological trough testosterone levels in that group. Irrespective to the roles of both LH and FSH in the control of spermatogenesis (reviewed in (McLachlan *et al.*, 2002a)), a clear correlation has been demonstrated

between incomplete suppression of spermatogenesis and failure in suppression of gonadotrophins with administration of combined progestogen/androgen regimes (Anderson *et al.*, 2002a). Indeed in this study, in group II there was a lesser and more inconsistent suppression of both LH and FSH, with levels being detectable for most of the treatment period which may contribute to ongoing spermatogenesis in that group. Furthermore, subphysiological testosterone concentrations as demonstrated in all mean trough levels in group II, in addition to raising concerns over hypogonadal side-effects, may also have contributed to a lesser suppression of the hypothalamic-pituitary axis in that group.

The treatment period in most other contraceptive studies has been for 6 months. The data presented in this chapter as in other recent studies (Gu *et al.*, 2003; Turner *et al.*, 2003) demonstrate the phenomenon of ongoing suppression of spermatogenesis up to the 48 week period. The cumulative effects of TD, observed in the increasing trough levels may have contributed to this ongoing suppression after the initial 6 month treatment period. In previous studies with testosterone esters resulting in supraphysiological androgen concentrations, there were concerns of adverse androgenic effects (Anderson *et al.*, 1996; Wu *et al.*, 1996) and also that high peripheral testosterone concentrations may contribute to paradoxical stimulation of spermatogenesis. Under conditions of high exogenous testosterone administration, the finding of similar intra-testicular and peripheral testosterone concentrations (McLachlan *et al.*, 2002b) may suggest reversal of the normal blood-testis gradient, and passive diffusion of testosterone from the circulation to the testis. Conversely in this study, at the testicular level under conditions of gonadotrophin withdrawal, the paradoxical

effects of stimulation of spermatogenesis from low intratesticular androgen (Zhang *et al.*, 2003), perhaps through up-regulation of testicular 5-alpha reductase (Mc Lachlan *et al.*, 2002) may be less with lower peripheral androgen levels. This may contribute to the high levels of spermatogenic suppression observed in this study.

'Rebound' of suppression of spermatogenesis from azoospermia was observed in this study in 3 individuals. This is not an uncommon feature of male hormonal contraceptive regimes and indeed very low sperm concentrations (Barfield *et al.*, 1979) and 'rebound' in efficacy studies (Gu *et al.*, 2003) has been associated with pregnancies. Although numbers are small, in this study, there is no consistency between gonadotrophin 'recovery' and 'rebound' of spermatogenesis; of the 3 individuals demonstrating 'rebound', one had incomplete suppression of FSH throughout treatment, another had detectable FSH 16 weeks prior to rebound, and in the final individual both FSH and LH were completely suppressed throughout the treatment period. It has been demonstrated from morphological studies in both animal models (Saito *et al.*, 2000; O'Donnell *et al.*, 2001) and in human testicular biopsies (Zhengwei *et al.*, 1998; McLachlan *et al.*, 2002b) that the important effects of gonadotrophin withdrawal are spermatogonial development and spermiation and that this may also be dependent on both FSH and androgen levels (Saito *et al.*, 2000; Narula *et al.*, 2001). Thus, release from later spermatogenic arrest may account for such rebound and also explain rapid inhibition that is observed in some individuals early in male contraceptive regimes.

During follow-up, a significant proportion of subjects exhibited a prolonged recovery of spermatogenesis, the longest period being 56 weeks. This has also been observed in

previous studies with progestogen/androgen combinations (Brenner *et al.*, 1977). The underlying reasons for this prolonged recovery are unclear but may either relate to the progestogen or the testosterone ester. Such prolonged recovery has not been observed in previous studies using a similar dose of desogestrel (Wu *et al.*, 1999; Anawalt *et al.*, 2000; Kinniburgh *et al.*, 2002). However, with the exception of the extension phase in the study by Kinniburgh *et al.*, these studies were for a duration of 24 weeks and not 48, and it cannot be ascertained that their follow-up was complete beyond the usual period of 16-24 weeks.

Although, the effects of gestogens on the hypothalamus and pituitary in men are unclear and shall be considered later in chapter 5, there is evidence to support a possible direct effect of gestogens on the testis. A non-classical progesterone receptor has been identified in spermatozoa (El-Hefwany *et al.*, 2000) and on rat Leydig cells (Rossato *et al.*, 1999) and progesterone has been seen to down-regulate LH receptor expression and function in vitro (El-Hefwany and Huhtaniemi, 1998). Desogestrel may also have a direct effect on Leydig cell steroidogenesis (Satyaswaroop and Gurpide, 1978) further decreasing intra-testicular testosterone concentrations and having an inhibitory effect on spermatogenesis. However, with a half-life of 23.8 hours (Bergink *et al.*, 1990), it is not likely that such mechanisms alone may account for the prolonged recovery of spermatogenesis.

Recovery data in previous studies of testosterone esters alone are incomplete. In the WHO study using TE alone (World Health Organisation Task Force on Methods for the Regulation of Male Fertility, 1996), although the mean time of recover to normal levels

was 16 weeks, 20% of subjects were lost to follow-up. Similarly, more recently in a large efficacy study in China administering TU alone, the recovery period was 12 months and no information was given on the follow up of the subjects who discontinued from the study (Gu *et al.*, 2003). The mechanisms of heterogeneity in response to hormonal regimes (already discussed in Chapter 1) have been subject of considerable debate (Handelsman *et al.*, 1995; Anderson *et al.*, 1996; Yu and Handelsman, 2001) and some subjects may exhibit a greater degree of suppression at earlier stages in the spermatogenic cycle such as type A to B spermatogonial development (Zhengwei *et al.*, 1998; O'Donnell *et al.*, 2001) and others more at later stages such as spermiation (Saito *et al.*, 2000) contributing to such a range in spermatogenic recovery. Thus the exact underlying mechanisms of this prolonged recovery are unclear, and warrant further investigation as this will clearly affect the acceptability of any potential male contraceptive.

Few subjects in this study reported hypoandrogenic side-effects. Incidentally, all discontinuations in the study were from group II, two of which experienced mood disturbance. This may be related to the overall lower mean testosterone concentrations in that group, however a placebo group would be required to infer more from these observations. The administration of desogestrel alone results in dose-dependent decline in SHBG (Cullberg, 1985) and this lowering of SHBG is observed with combined administration with exogenous androgen in this study as in others (Wu *et al.*, 1999; Anawalt *et al.*, 2000; Kinniburgh *et al.*, 2002). Under such conditions of lowered SHBG, free or bio-available testosterone would increase. Although mean bioavailable testosterone concentrations remain within the normal range, mean trough concentrations

are below normal range in group II for the duration of the treatment period, reflecting the 'under-replacement' in that group. A smoother pharmacokinetic profile is obtained with testosterone pellets which have near zero-order release (Handelsman *et al.*, 1990), a dose of 400mg every 12 weeks in contraceptive studies (Anderson *et al.*, 2002a; Kinniburgh *et al.*, 2002) maintaining testosterone concentrations within physiological range. This approach shall be considered later in Chapter 4.

As with other progestogen and testosterone combinations, weight gain, an increase in haemoglobin and haematocrit and a fall in HDL-C are observed in this study. It is interesting that the increases in body weight, and haemoglobin and haematocrit were greater and significant in group I, the group receiving a higher dose of TD. Although demonstrating comparatively greater selectivity for the progesterone receptor than other synthetic gestogens (Phillips *et al.*, 1990), etonogestrel still binds to the androgen receptor and may therefore contribute to these androgenic effects. With low physiological androgen levels in this study and the avoidance of supraphysiological peaks in both groups, it would be difficult to attribute these effects entirely to TD. In studies of desogestrel when given in combination with oestrogen containing oral contraceptive pills, desogestrel has a minimal impact on weight and in fact is associated with an increase in HDL-C (Archer, 1994). However, in combination with testosterone, it has been demonstrated that weight gain and decline in HDL-C concentrations are dependent on both the dose of desogestrel and testosterone (Anawalt *et al.*, 2000). Therefore, it is likely that both may contribute to this effect and this raises concern with the association between decreased serum HDL-C and cardiovascular risk (reviewed in (Wu and von Eckardstein, 2003)). Few studies have investigated the specific body

compositions that are affected by exogenous steroid administration in male contraceptive regimes. In a cohort of subjects in the WHO study, body composition was analysed and the administration of TE alone resulted in an increase in fat free/lean body mass and decline in fat mass (Young *et al.*, 1993). Similarly, in combination with the gestogen levonogestrel, fat free mass increases with an attenuation of the decline in fat mass observed with exogenous androgen alone (Herbst *et al.*, 2003). Our results support these data, with a significant increase in fat free mass alone in group I during the combined administration of testosterone and etonogestrel.

Thus the combination of oral etonogestrel and intramuscular testosterone decanoate results in profound suppression of both gonadotrophins and spermatogenesis. Group I exhibits a higher degree of efficacy in terms of both suppression of gonadotrophins and spermatogenesis with all subjects eventually achieving azoospermia in that group. By 24 weeks of treatment, all subjects had sperm concentrations of $< 1\text{M/ml}$, which in recent efficacy studies has been used as an acceptable threshold for entry to the efficacy phase (Turner *et al.*, 2003; Gu *et al.*, 2003). Delay to achieve this level of spermatogenic suppression may be viewed as an impediment to the acceptability of a potential contraceptive product (Martin *et al.*, 2000). Clearly the dose of TD in group II is suboptimal, with persistently low mean trough testosterone concentrations and a lesser suppression of spermatogenesis. Similar to previous combined androgen-gestogen regimes, a decline in HDL-C and increase in body weight were observed. A higher efficacy in suppression of spermatogenesis and gonadotrophins is reported in recent studies using etonogestrel implants delivering lower equivalent daily doses of

etonogestrel (Anderson *et al.*, 2002a), with reduced non-reproductive side-effects. This will be the subject of investigation in the following chapters.

CHAPTER 3

A MULTI-CENTRE STUDY INVESTIGATING SUBCUTANEOUS ETONOGESTREL IMPLANTS WITH INJECTABLE TESTOSTERONE DECANOATE AS A POTENTIAL LONG-ACTING MALE CONTRACEPTIVE.

3.1 Introduction

The data presented in this chapter are based on a Phase IIb multi-centre trial investigating the combination of etonogestrel implants with testosterone decanoate as a male hormonal contraceptive (funded by Organon NV). I was responsible for the recruitment of subjects, and management of the Edinburgh cohort, and analysis and reporting of data from all centres.

Etonogestrel, the active metabolite of desogestrel, is now licensed as a long-acting implant preparation for use as a female contraceptive. In addition to convenience and higher levels of compliance, administration as an implant may confer advantages over an oral preparation allowing dose-sparing, and avoiding liver exposure by bypassing first pass metabolism, thus minimising adverse metabolic effects. Indeed, this has been demonstrated in a recent study using two 68mg etonogestrel implants with depot testosterone (Anderson *et al.*, 2002a). Despite using lower equivalent doses of progestogen in comparison to oral desogestrel, (Kinniburgh *et al.*, 2002) similar efficacy in suppression of spermatogenesis was observed with reduced non-reproductive effects. In that study, suppression of spermatogenesis was greater with 2 rods than with 1 (75 vs 64% azoospermia). We therefore hypothesised that suppression of spermatogenesis may be improved by further increasing this dose by 50% and two larger rods (each containing 102mg etonogestrel) were administered in this study.

We also further explored the optimal dose of the same androgen investigated in chapter 2, by 3 administration patterns: 400mg every 4 weeks (group I) and 6 weeks (group II) and 600mg every 6 weeks (group III).

The primary objective of this study was to assess the effects of this combination on the suppression of spermatogenesis in the three treatment groups. Secondary objectives included evaluation of its effect on the suppression of gonadotrophins, the pharmacokinetics of the regime, assessment of its effect over the period of 48 weeks and monitoring of its safety.

3.2 Subject and methods

Subjects

One hundred and thirty subjects were recruited from six centres as follows: Münster (25), Edinburgh (20), Manchester (21), Turku (20), Helsinki (21) and Seattle (23). Men were aged 18 to 45 years (mean age 31 yr). The majority of subjects were Caucasian (124, 95.4%), the remainder of Asian (3, 2.3%) or other origin (3, 2.3%). The inclusion criteria included age ≥ 18 years and ≤ 45 years; mentally and physically healthy; body mass index (BMI) ≥ 18 and ≤ 32 kg/m²; normal semen analysis on two occasions (examination within 60 minutes, based on WHO criteria (WHO, 1999) for sperm concentration and WHO criteria or local reference ranges for sperm motility and morphology); normal hormone (FSH, LH, T) levels based on local reference ranges; willing to provide written informed consent. Men in a sexual relationship at study inclusion had to be willing to use a reliable form of contraception. Each subject gave informed written consent according to the Declaration of Helsinki Guideline for Good

Clinical Practice. Ethical approval was received from each centre's local Ethical Review Committee.

Medication

Etonogestrel implants were 6cm long and contained 102mg etonogestrel (Organon N.V., Oss, The Netherlands) and were inserted under local anaesthetic under the skin of the medial aspect of the non-dominant upper arm and removed following the 48 week treatment period. Testosterone decanoate (3-oxo-androst-4-en-17 β -yl decanoate) at a concentration of 200mg/ml was administered by deep intramuscular injection on the day of etonogestrel implant insertion. Subjects re-attended every 4 or 6 weeks (+/- 3 days) thereafter, depending on the treatment group, for subsequent injections.

Study Design (see Tables 3.1 and 3.2)

The study was an open-labelled randomised multi-centre (Edinburgh UK, Manchester UK, Helsinki Finland, Turku Finland, Münster Germany and Seattle USA) trial investigating the suppressive effects of etonogestrel subcutaneous implants with injectable testosterone decanoate on spermatogenesis. The study also aimed to investigate the suppressive effect of this regime on gonadotrophins and the safety and pharmacokinetics of this regime. Subjects were randomised into 3 treatment groups. All groups received 2 etonogestrel implants. Group I received 400mg TD every 4 weeks, group II 400mg TD every 6 weeks and group III 600mg TD every 6 weeks. Subjects were reviewed every 4 weeks in the first 24 weeks of the treatment phase and during recovery. During weeks 24 to 48 subjects attended every 4 or 6 weeks depending on the treatment group. At each visit, subjects submitted a semen sample

Week	Pre-treatment										Treatment									
	-4	0	1	2	4	8	12	13	14	16	20	24	28	32	36	40	44	48		
Physical exam	✓						✓					✓			✓			✓		
Routine labs ¹ & PSA	✓						✓					✓			✓			✓		
ENG	✓		✓	✓	✓	✓	✓					✓		✓	✓			✓		
T/DHT/SHBG	✓		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓			✓		
FSH/LH	✓				✓		✓					✓			✓			✓		
Semen sample	✓			✓	✓	✓	✓			✓	✓	✓	✓	✓	✓	✓	✓	✓		
Testes & prostate vol	✓											✓						✓		
DISF-SR/mood	✓						✓					✓			✓			✓		
Implant insertion /removal		✓																✓		
TD injection		✓			✓	✓	✓			✓	✓	✓	✓	✓	✓	✓	✓			

Table 3.1: Flow chart of subject assessments in (pre)treatment phase of Group I receiving TD 400mg i.m. every 4 weeks.
¹Includes haematology, biochemistry, liver function tests, fasting and fasting lipids. PSA was drawn before prostate examination

Week	Pre-treatment										Treatment									
	-4	0	1	2	4	6	8	12	13	14	16	18	20	24	30	36	42	48		
Physical exam	✓							✓						✓		✓		✓		
Routine labs ¹ & PSA	✓							✓						✓		✓		✓		
ENG	✓		✓	✓	✓		✓	✓	✓	✓	✓	✓		✓		✓		✓		
T/DHT/SHBG	✓		✓	✓	✓	✓		✓	✓	✓	✓	✓		✓		✓		✓		
FSH/LH	✓				✓	✓		✓						✓		✓		✓		
Semen sample	✓				✓		✓	✓			✓		✓	✓	✓	✓	✓	✓		
Testes & prostate vol	✓													✓				✓		
DISF-SR/mood	✓							✓						✓		✓		✓		
Implant insertion /removal	✓																	✓		
TD injection	✓					✓		✓				✓		✓	✓	✓	✓			

Table 3.2: Flow chart of subject assessments in (pre)treatment phase of Groups II and III receiving TD 400mg and 600mg i.m. respectively every 6 weeks.
¹Includes haematology, biochemistry, liver function tests, fasting and fasting lipids. PSA was drawn before prostate examination

and safety assessments were performed checking routine lab parameters including PSA, inspecting the implant site, recording adverse events and any concomitant medications. Physical examination was performed every 12 weeks (with andrological examination assessing testes and prostate assessment by digital examination or transrectal ultrasonography at weeks 24, 48 and final assessment). Venepuncture was performed for hormone measurements. Additional samples were taken at weeks 1, 2 after the first injection and 13 and 14 after the second (6 week) or third (4 week) injection to assess testosterone pharmacokinetics. Etonogestrel levels were assessed at weeks 1,2,4 and 8 and at all extensive assessment days. During the follow-up phase subjects attended every 4 weeks until week 16 at which stage if sperm concentration was greater than $20 \times 10^6/\text{ml}$ they underwent final assessment or they continued until week 24. Any subject unrecovered at week 24 of the recovery phase entered an extended phase of follow up of indefinite duration.

Semen analysis

Semen samples were submitted after 2 to 7 days of abstinence and assessed for semen volume, sperm concentration, morphology and motility by WHO criteria (WHO, 1999). Motility was assessed within 60 minutes of ejaculation. For morphology and motility local reference ranges were used to determine normal ranges. Azoospermia was confirmed by centrifugation of the entire ejaculate and systematic examination of the pellet. Semen samples were performed in individual centres using comparable methodologies and were externally assessed for Quality Control by Organon NV. A study examining the variation between laboratories with semen analysis demonstrated consistency in the assessment of sperm concentration and semen volume when using

similar methodologies, despite significant inter-individual variation (Jorgensen *et al*, 1997). The assessment of sperm motility and morphology are less consistent, neither of which were used in the end-points of this study.

Assays

Blood samples were separated by centrifugation and serum stored at -20°C prior to shipping to a central laboratory (Organon) for assay. Assay methodology, sensitivities, intra and interassay coefficients of variation are as described in Chapter 2.

Behavioural assessment

Sexual function was investigated pre-treatment, at 3 to 4 monthly intervals during treatment and at final assessment by means of the Derogatis Interview for Sexual Functioning-Self Report (DISF-SR) (Derogatis, 1997). Questions on mood and local tolerance of injections were also assessed by an unvalidated questionnaire.

Statistical Analysis

Statistical analysis in this chapter was performed by Emmanuel Aris at Organon NV. Frequencies of subjects with suppression of sperm concentration to a specified level and a certain time point were compared by means of a Fisher's exact test with Bonferroni correction. Survival analyses were performed and because of departure from the proportional hazards assumption, Log-Rank tests were used for comparison. Mean values of sperm concentrations, hormones, biochemistry, haematology and physical parameters were analysed by repeated measure ANOVA and paired t-tests using Tukey's multiple comparison procedure. P-values presented for hormones,

biochemistry, haematology and physical parameters were not corrected for multiple testing, however multiplicity was taken into account by regarding a result statistically significant if $P < 0.0001$ (which would correspond to Bonferroni corrected P-values below 0.05). Values were expressed as the arithmetical mean \pm SEM. Hormones values below the detection level were allocated the value of half of the lower limit of detection.

3.3 Results

Subjects

Pretreatment values for the subjects in each group is demonstrated in Table 3.3. There were no significant differences in age, BMI, sperm concentration and LH, FSH and testosterone concentrations between the 3 treatment groups.

One hundred and thirty subjects were randomised to the 3 groups as follows: group I (n=42), II (n=46) and III (n=42). Five subjects in group III were treated erroneously with 400mg TD instead of 600mgTD and therefore were analysed in group II, resulting in 42, 51 and 37 subjects in the respective groups. In total, 119 subjects completed 24 weeks of treatment and 110 subjects completed the treatment period (84.6%): 33 subjects in group I (78.6%), 43 subjects in group II (84.3%) and 34 subjects in group III (91.9%). Overall, compliance with study medication was good with 100% compliance with TD injections in group II and near 99% compliance in the other 2 treatment groups.

Sperm Concentrations

Whilst it has been established that there are regional differences in sperm concentrations (Jorgensen *et al*, 2001), there were no differences between centres in baseline sperm concentrations. All men demonstrated a profound suppression of spermatogenesis in this study (Figure 3.1). By week 24 azoospermia was achieved in 28 (71.8%), 25 (55.6%) and 24 (68.6%) subjects in groups I, II and III respectively (Figure 3.2, Table 3.4). The extent of suppression in groups I and III at week 24 were similar and greater

Table 3.3: Pre-treatment values of subjects in the 3 treatment groups. Data presented as mean±sem.

Pre-treatment value	Group I (n=42)	Group II (n=51)	Group III (n=37)
Age (yr)	30.7±0.9	31±0.8	31.5±1.1
BMI (kg/m ²)	24.6±0.4	24.7±0.4	25.1±0.5
LH (IU/L)	3.7±0.3	3.6±0.2	3.7±0.3
FSH (IU/L)	3.5±0.3	3.3±0.2	3.6±0.3
Testosterone (nmol/L)	19.2±1.3	18.7±0.7	20.5±1.3
Sperm concentration (M/ml)	72.6±6.6	90.6±7.9	75.2±7.7
Sperm concentration (M/ml)*	59.6 (21.8-247.5)	73.8 (31.9-251.5)	55.1 (19.4-180.3)

*Sperm concentration presented as median (range).

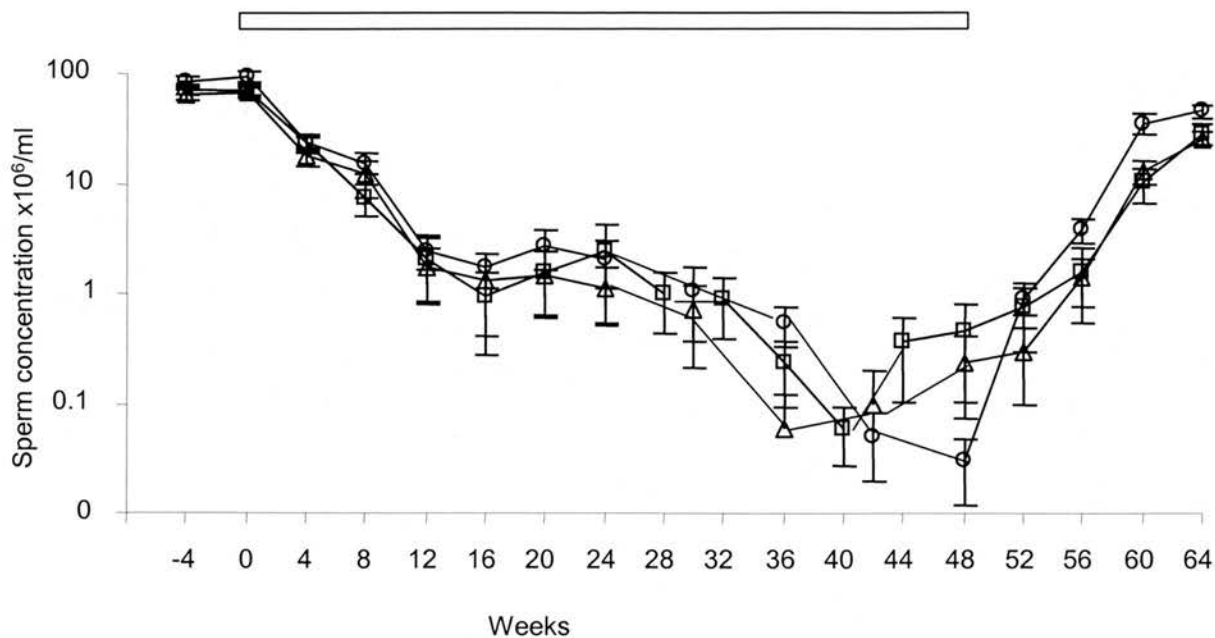


Figure 3.1. Sperm concentrations during etonogestrel implants/TD treatment and first 16 weeks recovery. Duration of treatment is indicated by the bars. Note log scale on the ordinate. Data presented as mean \pm sem.

- Group I (400mgTD/4 wks)
- Group II (400mg TD/6 wks)
- △ Group III (600mg TD/6wks)

Table 3.4: Percentage of subjects suppressed to sperm concentration targets, receiving treatment with etonogestrel implants and i.m TD at the doses indicated.

Treatment group		% of subjects reaching the suppression targets				
	Week	n	Azoospermia	<0.1 M/ml	<1M/ml	<3M/ml
400/4-week	Week 8	39	10.3	43.6	56.4	69.2
	Week 16	37	48.6	81.1	89.2	91.9
	Week 24	39	71.8	87.2	89.7	99.7
	Week 48	31	80.6	93.5	93.5	93.5
400/6-week	Week 8	49	4.1	22.4	40.8	51.0
	Week 16	44	38.6	61.4	81.8	84.1
	Week 24	45	55.6	71.1	82.2	88.9
	Week 48	40	77.5	92.5	100	100
600/6-week	Week 8	36	11.1	38.9	50.0	63.9
	Week 16	34	52.9	85.3	88.2	94.1
	Week 24	35	68.6	80.0	88.6	91.4
	Week 48	33	84.8	90.9	93.9	93.9

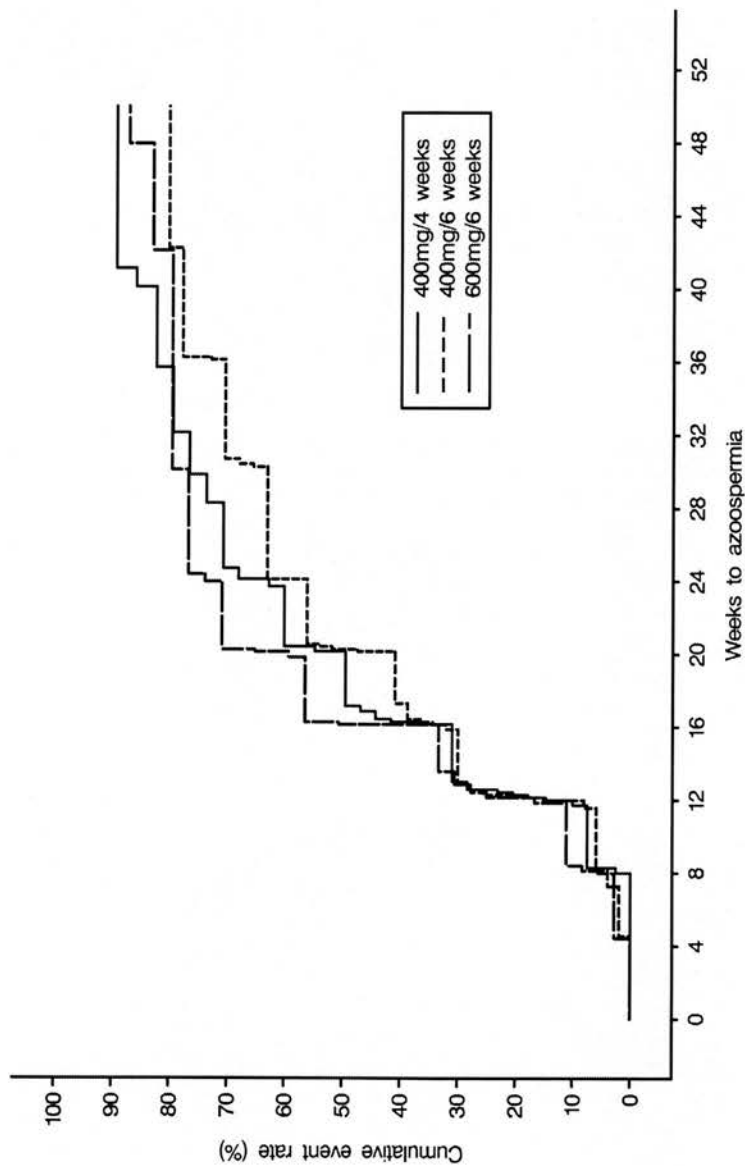


Figure 3.2. Achievement of azoospermia during etonogestrel/TD treatment. This is a cumulative event rate assessed by Kaplan-Meier estimation. Treatment groups are indicated in legend.

than suppression demonstrated by group II, although there was no statistically significant difference between suppression in groups at weeks 16 or 24. At week 24, sperm concentrations had fallen to <1M/ml in 90% (group I), 82% (group II) and 89% (group III) of subjects (Figure 3.3). Four subjects in group I, 5 in group II and 3 in group III maintained sperm concentrations of > 3M/ml at week 24; all but 4 of these proceeded to concentrations close to azoospermia by week 48. Sperm concentrations further decreased in all groups after week 24, with azoospermia being achieved in 81% (group I), 78% (group II) and 85% (group III) of subjects by the end of the treatment period. Once azoospermic, this was maintained at all subsequent visits in 56% (group I), 72% (group II) and 80% (group III) of subjects. Using the threshold of <1M/ml, faster suppression of spermatogenesis was observed in groups I and III, the median number of days to reach this threshold being 59 (group I), 84 (group II) and 61 (group III). However, suppression to azoospermia occurred in a similar time for all three groups, median number of days to reach this being 114 (group I), 118 (group II) and 113 (group III). By week 24 of follow-up, (ITT group) 77% (group I), 82% (group II) and 83% (group III) of subjects had reached (at least one) normal sperm concentrations by WHO criteria. Recovery was faster in group II (400mg/6 weeks) than in groups I and III (at week 24: $p=0.01$) with a median time to recovery of approximately 130 days in all treatment groups (Figure 3.4). Excluding the subjects who discontinued follow-up prematurely (3 subjects), the remainder (group I (8), group II (7), group III (6)) entered a period of extended follow-up. After that time, 92.3% (group I), 94.1% (group II) and 94.4% (group III) had at least one normal sperm concentration by WHO criteria.

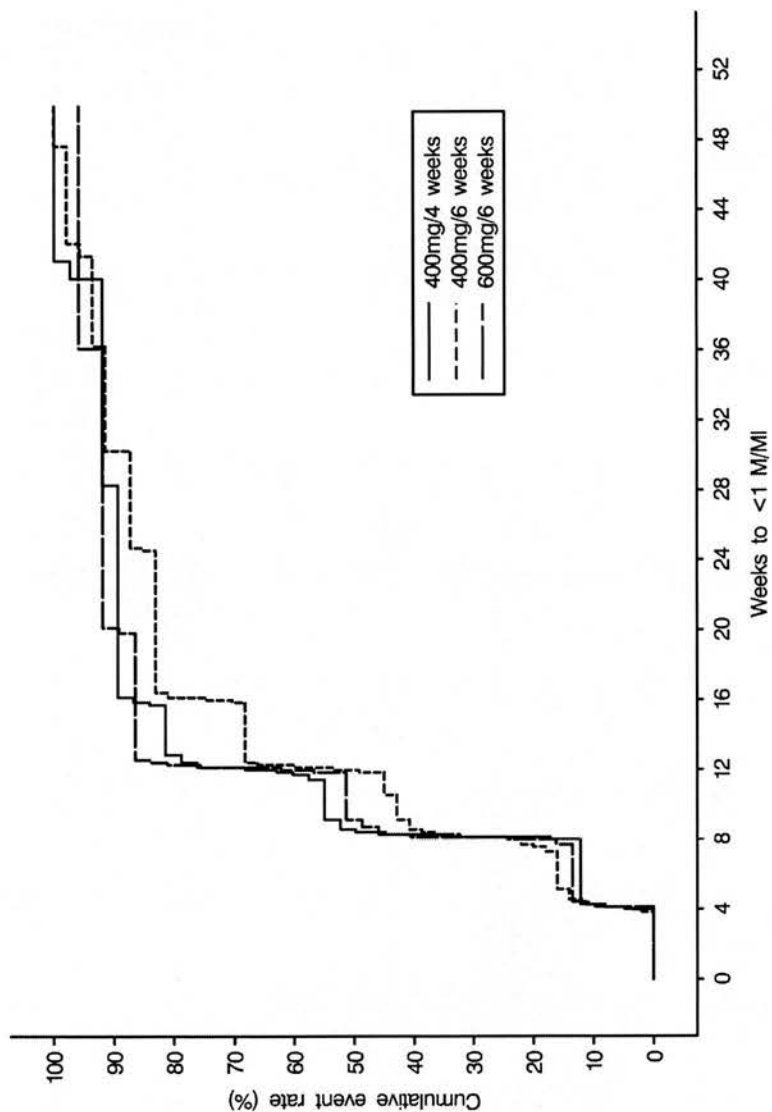


Figure 3.3. Achievement of sperm concentrations of < 1million/ml with etonogestrel/TD treatment. This is a cumulative event rate assessed by Kaplan-Meier estimation. Treatment groups indicated in legend.

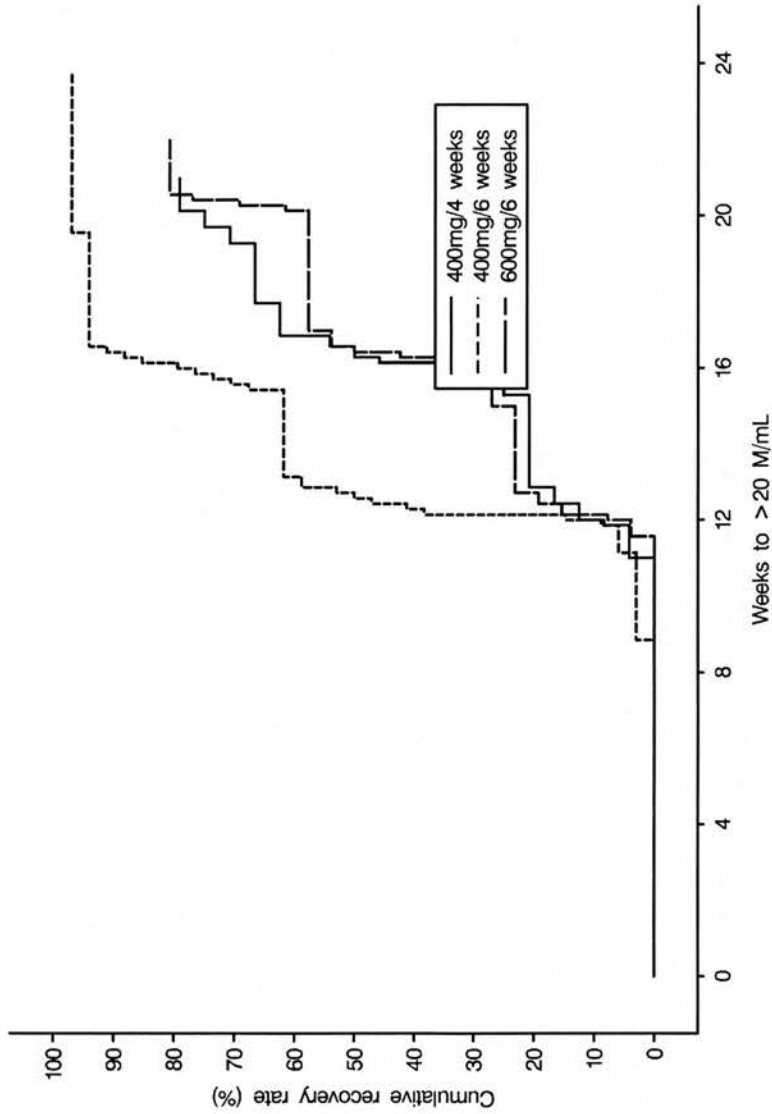


Figure 3.4. Achievement of recovery of spermatogenesis to concentrations of >20 million/ml following etonogestrel/TD treatment. This is a cumulative event rate assessed by Kaplan-Meier estimation. Treatment groups as indicated in legend.

Reproductive Hormones

Profound suppression of both LH and FSH was observed in all three treatment groups (Figure 3.5). Gonadotrophin levels were suppressed to the level of detection by week 4 in the majority of subjects and remained suppressed throughout the treatment period. Suppression was however less consistent in the 400/6wk group (group II) with more frequent fluctuation or 'escape' being observed. Recovery of both LH and FSH was faster in group II than in I and III ($p < 0.05$ at 4 weeks of follow-up). There were no statistically significant differences between groups at the end of follow up and comparing final visit with pre-treatment gonadotrophin values.

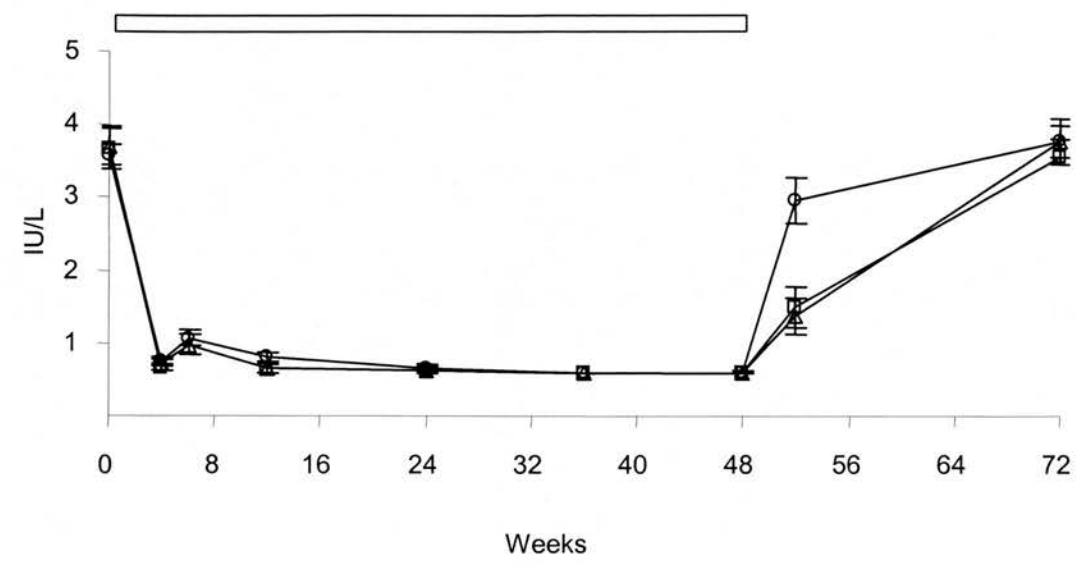
Testosterone

Fluctuations in testosterone concentration were observed, in keeping with the scheduling of testosterone decanoate injections (Figure 3.6a). Peak testosterone concentrations remained within the physiological range for all groups throughout the treatment period. Mean trough testosterone concentrations gradually increased over the time-course of the study, and were initially sub-physiological in all 3 treatment groups (Figure 3.6b). In group II, mean trough testosterone concentrations remained below physiological range until week 36 whereas in group I mean trough testosterone concentrations were in the normal range between week 8 and 12 and in group III between weeks 12 and 18.

Other hormones

Peak mean etonogestrel concentrations were observed 1 week after insertion of the implants with similar concentrations in all three treatment groups of approximately 800-

(a)



(b)

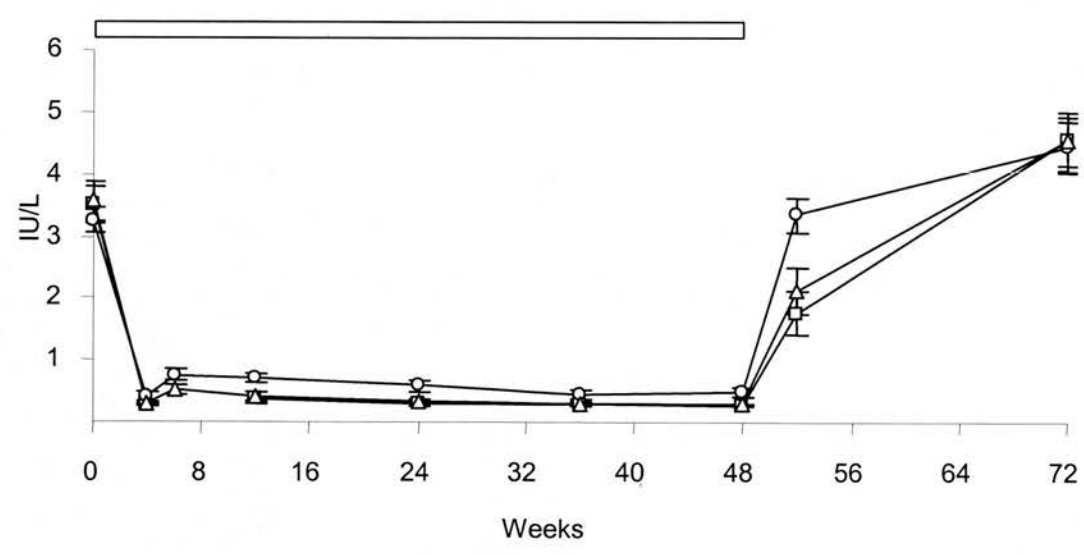
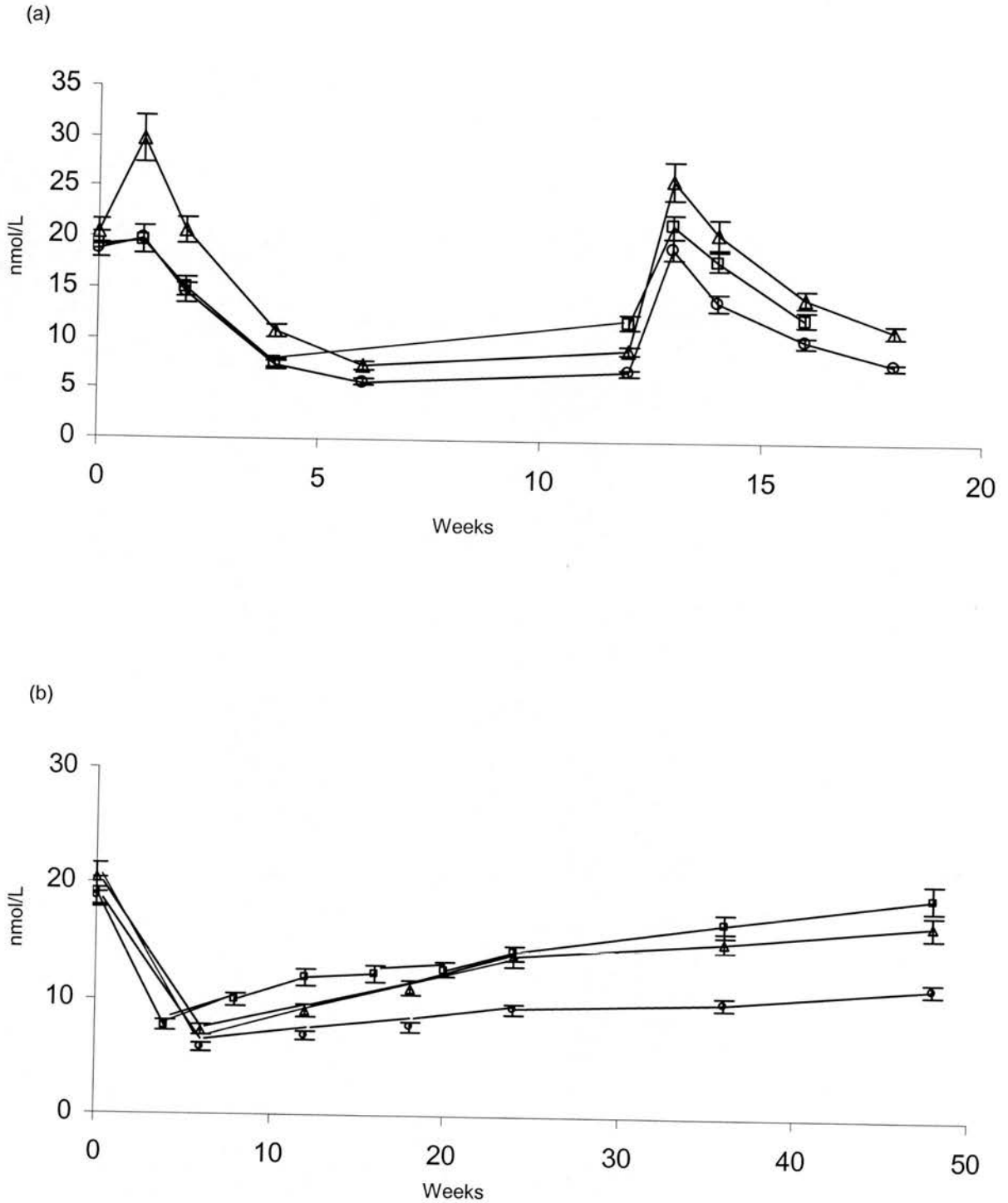


Figure 3.5: (a) Serum LH and (b) FSH concentrations during etonogestrel/TD treatment and recovery. Data presented as mean +/- sem. □ Group I (400mg TD/4wks) ○ Group II (400mg TD/6wks) △ Group III (600mg TD/6wks)



900pg/ml. Thereafter, there was a gradual decline in levels to approximately 300pg/ml after 48 weeks of treatment.

Biochemistry & Haematology

Serum SHBG concentrations decreased within 8 weeks of treatment in all 3 treatment groups remaining so until the end of treatment and returning to baseline concentrations thereafter.

Mean haemoglobin concentrations increased in all groups during treatment (reaching statistical significance in group I at 48 weeks, $p<0.0001$), returning to baseline levels after follow-up. An increase in mean haematocrit was observed in groups I and III during treatment returning to baseline levels thereafter. There was a decline in total cholesterol and HDL-C in all groups (less in group II) returning to baseline levels following treatment (Table 3.5).

Physical examination & Behaviour

Testicular volume decreased by approximately 25% in all groups during treatment, returning to pre-treatment volumes during the recovery phase (Table 3.6). Inter-observer variation was however unaccounted for. A more accurate method may have been the assessment of testes volumes in all centres by ultrasonography (Behre *et al*, 1989). There was no significant change in prostate volume, PSA or blood pressure throughout the study period. There was a slight increase in weight of 5% (group I), 3.5% (group II), and 5% (group III) that did not reach statistical significance. There was no significant change in overall mood scores across treatment groups throughout the study. There were no changes in the overall scores from the Derogatis interview for

Table 3.5: Haematological and biochemical parameters during treatment with ENG implants and TD as indicated and at final visit. Data presented as mean±sem.

	Group I (400mg/4wk)				Group II (400mg/6wk)				Group III (600mg/6wk)			
Week	0	24	48	final	0	24	48	final	0	24	48	final
Hb (g/L)	151.5±1.1	156.1±1.3	158.7±1.4 ^a	154.6±1.2	151.0±1.3	152.1±1.3	154.5±1.2	152.8±1.3	150.4±1.4	154.2±1.4	156.9±1.4	151.3±1.3
Hct	0.45±0.01	0.46±0.01	0.47±0.01	0.45±0.01	0.45±0.01	0.45±0.01	0.45±0.01	0.45±0.01	0.44±0.01	0.45±0.01	0.46±0.01	0.44±0.01
Cholesterol (mmol/L)	4.7±0.1	4.4±0.2	4.5±0.2	4.8±0.2	4.6±0.1	4.6±0.1	4.5±0.1	4.8±0.1	4.7±0.1	4.6±0.2	4.5±0.2	4.8±0.1
HDL-Chol (mmol/L)	1.4±0.1	1.1±0.1	1.1±0.1	1.4±0.1	1.4±0.1	1.3±0.1	1.2±0.1	1.3±0.1	1.4±0.1	1.2±0.1	1.2±0.1	1.3±0.1
LDL-Chol (mmol/L)	2.8±0.1	2.7±0.1	2.9±0.1	2.9±0.1	2.7±0.1	2.8±0.1	2.8±0.1	2.9±0.1	2.7±0.1	2.8±0.1	2.8±0.1	2.9±0.1
Triglycerides	1.2±0.1	1.2±0.1	1.1±0.1	1.2±0.1	1.1±0.1	1.1±0.1	1.1±0.1	1.2±0.1	1.2±0.1	1.2±0.1	1.3±0.1	1.3±0.1
SHBG (nmol/L)	32.6±2.0 ^a	24.9±2.3	25.4±2.7	31.2±2.1	37.1±2.1	27.1±1.6 ^a	27.4±1.8	33.3±2.0	38.6±2.6	26.7±2.0 ^a	26.1±2.3	37.7±2.8
ALT (U/L)	25.1±1.9	25.4±2.0	26.0±2.5	29.3±2.2	23.0±1.8	24.5±2.1	24.2±2.4	29.4±2.1	23.2±1.6	24.9±1.9	24.9±2.0	31.4±3.2
AST (U/L)	22.0±1.4	22.82.4	20.8±1.3	23.5±1.3	23.4±1.4	21.8±1.3	21.4±1.2	24.4±1.3	22.6±1.3	21.2±1.3	21.1±1.3	26.3±1.8
GGT (U/L)	21.7±1.6	27.1±2.9	27.8±2.9	27.5±2.8	23.7±2.1	29.2±3.3	28.1±2.7	28.4±3.1	20.5±1.6	26.2±2.1	28.9±2.8	27.4±3.0
Bilirubin (µmol/L)	15.0±1.0	14.9±1.2	17.3±1.3	13.8±1.0	14.5±0.9	13.9±0.8	15.9±1.1	13.8±0.9	15.9±1.3	15.5±1.4	15.8±1.3	15.2±1.4

Data presented as mean ± sem. ^a Statistically significant result p<0.0001. There were no statistically significant differences between the 3 groups at any treatment point.

Table 3.6: Clinical parameters during treatment and final visits for all 3 groups

Week	Group I (400/4wk)					Group II (400/6wk)					Group III (600/6wk)				
	0	24	48	final	0	24	48	final	0	24	48	final	0	24	48
Weight (kg)	79.0±1.6	81.7±1.5	82.9±1.7	80.0±1.4	79.4±1.7	82.0±1.8	82.0±1.9	81.5±1.6	79.3±1.7	83.3±1.7	83.2±1.9	81.4±1.9			
Prostate vol	18.6±1.0	19.6±1.0	19.6±1.3	18.2±1.0	17.7±1.0	18.6±1.2	17.9±0.7	17.1±0.9	19.2±1.1	19.6±1.0	18.6±1.1	17.3±0.8			
PSA (ng/ml)	0.9±0.1	1.0±0.1	1.1±0.1	1.0±0.1	0.7±0.1	0.8±0.1	0.7±0.1	0.7±0.1	0.8±0.1	0.9±0.1	0.8±0.1	0.8±0.1			
Mean Testicular Volume (ml)	22.6 ±0.7	17.0±0.8 ^a	16.4±0.9 ^a	22.4±0.9	24.9±0.7	18.7±0.8 ^a	17.8±0.8 ^a	24.3±0.8	24.8±0.7	17.4±0.9 ^a	16.2±0.9 ^a	23.0±0.8			
Overall DISF score	103.2±2.4	103.0±3.0	102.7±3.4	97.8±2.7	101.7±2.2	102.3±2.8	100.8±2.7	96.3±3.1	103.5±2.5	107.3±3.0	107.9±3.3	103.3±3.2			

Data presented as mean±sem. ^a Statistically significant, p<0.0001.

sexual function throughout treatment or follow-up. Similarly, there were no differences in mean subscores for different DISF functions.

Discontinuations and side-effects

Overall, 20 subjects discontinued the study in respective groups as follows: (9 (group I), 8 (group II), 3 (group III)). The most frequent reason for discontinuation was adverse events (14 subjects, 10.8%), other reasons being non-compliance with the protocol or moving area. In group II only one subject discontinued due to an adverse event, which was erectile dysfunction. One other subject discontinued in group I due to impotence. Other adverse events that led to discontinuation were: aggressive reaction (with or without nervousness or emotional lability) (3), implant complication (2), depression (2), emotional lability (2), arthralgia (1), laryngitis (1) and myocarditis (1).

Among the possible side-effects that did not lead to discontinuation, acne and increased sweating, mood changes, weight increase and mild reactions related to implant insertion or removal were most frequently reported. Most of the latter events concerned mild itching, pain or swelling.

3.4 Discussion

The combination of etonogestrel implants and injectable testosterone decanoate resulted in profound suppression of spermatogenesis, comparable to other approaches using a combination of androgen and progestogen for male hormonal contraception (Anderson and Baird, 2002; Nieschlag *et al.*, 2003). In this study comparatively large numbers of subjects were used for the longer treatment period of 48 weeks which has only been investigated in few studies to date (Gu *et al.*, 2003; Turner *et al.*, 2003). Overall, azoospermia was achieved by 24 weeks in 55-72% of subjects increasing to 78-85% of subjects by week 48. In the only contraceptive efficacy study to date using an androgen-progestogen combination, no pregnancies were reported with 426 months of contraceptive exposure in 51 men using the suppression threshold of 1M/ml (Turner *et al.*, 2003). Using the combination of etonogestrel implants with TD, these levels were achieved in approximately 90% of subjects by week 24, demonstrating that it may have potential as an efficacious contraceptive method.

While all men demonstrated suppression of spermatogenesis, there were nonetheless differences in the rate and the degree of suppression between treatment groups. It is possible that periods of abstinence may have affected rates of azoospermia. However this data was not collected and did not form part of the analysis. At week 24, suppression to azoospermia was slower in group II (56%) than in groups I (72%) and III (69%) although this difference was less by the end of the treatment period (83% group I; 78% group II; 89% group III). There was also a lesser suppression of gonadotrophins in group II, with a greater fluctuation than in the other 2 groups. This may be attributed to the less frequent administration of testosterone in that group. Indeed, trough

testosterone levels in group II remained sub-physiological until week 36, and the ongoing suppression in the latter half of the study may be related to the normalised trough testosterone levels. In other studies of similar length, further suppression is observed beyond the length of the spermatogenic cycle. Evidence in both primate models (Saito *et al.*, 2000; O'Donnell *et al.*, 2001) and humans (Zhengwei *et al.*, 1998; McLachlan *et al.*, 2002b) suggests that the sites of inhibition under such conditions of gonadotrophin withdrawal are both at the earlier stages of spermatogonial Type A to Type B progression and later spermiation. Continuing inhibition of spermatogenesis at earlier stages in the cycle may contribute to this ongoing suppression. The lesser suppression of spermatogenesis in group II is also reflected in the quicker recovery in that group.

Although the testosterone dosages used in the current study maintain peak testosterone concentrations below the supraphysiological range, trough concentrations showed a continuing slight increase during the length of the treatment period. While it therefore appears that yet longer studies are required to fully investigate the pharmacokinetics of this preparation, the present data clearly indicate that TD has advantages compared to previously available testosterone preparations. Similarly, testosterone undecanoate (TU) has improved pharmacokinetics compared to testosterone enanthate (Chen *et al.*, 1991; Li *et al.*, 1994). In studies with a repeated injection schedule of TU (Nieschlag *et al.*, 1999; Zhang *et al.*, 1999; Gu *et al.*, 2003; Gu *et al.*, 2004), similar cumulative effects were also observed. Previous studies in which peak testosterone levels are not assessed (Zhang *et al.*, 1999) may underestimate the total exposure to testosterone. These fluctuations in serum testosterone concentration do not however appear to be

reflected in the adverse events that did not differ significantly between groups. The slow improvement of testosterone preparations has been a significant barrier to the development of hormonal male contraception, and it appears that these newer preparations offer considerable advantages. The other long-acting testosterone preparation, subcutaneous pellets (Handelsman *et al.*, 1990), also have high efficacy in the context of male contraception, their zero-order release allowing dose-sparing (Handelsman *et al.*, 1992; McLachlan *et al.*, 2000). No studies have yet directly compared these preparations, but the considerable improvements in testosterone delivery exemplified by this TD preparation now make detailed investigation of the testosterone regimen of importance.

A proportion (16%) of men in all 3 groups entered a period of prolonged follow-up with delayed recovery in spermatogenesis. This has also been observed in previous studies with gestogen/androgen combinations (Brenner *et al.*, 1977). The underlying reasons for this prolonged recovery are unclear but may either relate to the gestogen or the testosterone ester. Such prolonged recovery has not been observed in previous studies using a similar dose of desogestrel (Wu *et al.*, 1999; Anawalt *et al.*, 2000; Kinniburgh *et al.*, 2002). However, with the exception of the extension phase in the study by Kinniburgh *et al.*, these studies were for a duration of 24 weeks and not 48, and it cannot be ascertained that their follow-up was complete beyond the usual period of 16-24 weeks. Although, the effects of gestogens on the hypothalamus and pituitary in men are unclear, there is evidence to support a possible direct effect of gestogens on the testis. A non-classical progesterone receptor has been identified in spermatozoa (El-Hefwany

et al., 2000) and on rat Leydig cells (Rossato *et al.*, 1999) and progesterone has been seen to down-regulate LH receptor expression and function in vitro (El-Hefwany and Huhtaniemi, 1998). Desogestrel may also have a direct effects on Leydig cell steroidogenesis (Satyaswaroop and Gurpide, 1978) further decreasing intratesticular testosterone concentrations and having an inhibitory effect on spermatogenesis.

Similar to other androgen/progestogen combinations (Bebb *et al.*, 1996; Wu *et al.*, 1999; Anawalt *et al.*, 2000), a decline in HDL-C and increase in body weight is observed in all groups. Although demonstrating comparatively greater selectivity for the progesterone receptor than other synthetic progestogens (Phillips *et al.*, 1990) etonogestrel still binds to the androgen receptor and may therefore contribute to these androgenic effects. With low physiological androgen levels in this study and the avoidance of supraphysiological peaks, it would be difficult to attribute these effects entirely to TD. In studies of desogestrel when given in combination with oestrogen containing oral contraceptive pills, desogestrel has a minimal impact on weight and in fact is associated with an increase in HDL-C (Archer, 1994). However, in combination with testosterone, it has been demonstrated that weight gain and decline in HDL-C concentrations are dependent on both the dose of desogestrel and testosterone (Anawalt *et al.*, 2000).

In conclusion, this study demonstrates profound suppression of spermatogenesis with the combination of etonogestrel implants and testosterone decanoate. Efficacy in spermatogenic suppression was greater in the groups I and III, than in group II which received 400mg TD every 6 weeks, indicating that this is a suboptimal regimen, and this

was supported by pharmacokinetic analysis. This is similar to the data presented in Chapter 2 with suboptimal suppression in the group receiving TD every 6 weeks. This combination is a valuable approach and demonstrates the potential of an implant preparation as a long-standing hormonal contraceptive for men. In the following chapter, this approach is further investigated using a different androgen delivery system.

CHAPTER 4

SUPPRESSION OF THE PITUITARY-TESTICULAR AXIS WITH ETONOGESTREL IMPLANTS AND TESTOSTERONE PELLETS

4.1 Introduction

As discussed in the preceding chapters of this thesis, promising results have been obtained using oral desogestrel, with high rates of azoospermia achieved in men from several ethnic backgrounds (Wu *et al.*, 1999; Anawalt *et al.*, 2000; Kinniburgh *et al.*, 2002). In the multicentre study in chapter 3, we considered etonogestrel, the active metabolite of oral desogestrel, which has recently been marketed in many countries as a long acting implant (Implanon ®, NV Organon, Oss, The Netherlands) providing 3 years of contraceptive efficacy in women. In this chapter the same implant preparation was investigated in combination with testosterone pellets as a potential long-acting male hormonal contraceptive.

Compared to oral administration, a long acting drug delivery system has advantages including dose-sparing and the avoidance of hepatic exposure to high doses, both of which may contribute to the reduction of unwanted adverse effects. Moreover it may be preferred by some individuals because of ease of compliance (Martin *et al.*, 2000a). Previous published data has reported investigation of 1 or 2 etonogestrel implants in combination with depot testosterone pellets (Anderson *et al.*, 2002a). Although profound suppression of spermatogenesis with minimal non-reproductive side effects was induced, azoospermia was achieved in only 64% and 75% of the 1 and 2 implant groups respectively. Etonogestrel implants release approximately 50µg/day, thus even

with two implants the daily dose is markedly lower than the optimally effective dose of 300µg desogestrel, which has approximately 80% oral bioavailability (Hasenack *et al.*, 1986).

There was therefore evidence for significant dose-sparing with the implant preparation, but as spermatogenic suppression was not complete in all men, it was hypothesised that the addition of a third etonogestrel implant may enhance this spermatogenic suppression. In this study, the duration of treatment was additionally extended to 48 weeks to investigate whether the steady decline in etonogestrel release from the implants would maintain suppression of gonadotrophins and thus spermatogenesis for that length of time, using the same testosterone regimen as previously used in the investigation of both oral desogestrel and etonogestrel implants (Anderson *et al.*, 2002a; Kinniburgh *et al.*, 2002). Although the equivalent dose of etonogestrel administration is the same as used in the multicentre study discussed in Chapter 3, this study complements these data, using a different preparation and method of androgen delivery, with testosterone pellets.

4.2 Subjects and methods

Subjects

Fifteen healthy men (mean age 31.6, range 18 – 37 years) were recruited from the same general population as previous studies (Anderson *et al.*, 2002a; Kinniburgh *et al.*, 2002). Inclusion criteria included age (18-45), mentally and physically healthy, BMI between 18-30 kg/m², normal pre-treatment FSH, LH and testosterone concentrations and routine haematological and biochemical analyses, 2 normal semen analyses according to

WHO criteria at least 2 weeks apart, and a normal physical and andrological examination. Pre-treatment sperm concentrations were greater than $20 \times 10^6/\text{ml}$ in all men, and motility and morphology were within normal ranges for the local population. Subjects provided written informed consent and the study had ethical approval from Lothian Reproductive Medicine Ethical Review Committee. The study was performed according to GCP guidelines.

Study Design and Medication (see Table 4.1)

This study was a single-group open investigation of the effects of etonogestrel implants with testosterone pellets. The number of subjects (15) was chosen following a power calculation demonstrating that this would give the study sufficient power to show a statistically significant difference in the primary end-point, the incidence of azoospermia with 3 implants in comparison to previous data using 2 implants, allowing for the possibility of 2 drop-outs. The duration of the treatment period was 48 weeks, with those subjects who were not azoospermic discontinuing treatment if they wished at 24 weeks. Following pre-treatment assessment, three implants each containing 68mg etonogestrel (Implanon, NV Organon, Oss, The Netherlands) were inserted subcutaneously in the medial aspect of the non-dominant upper arm to all subjects. All subjects additionally received 400mg testosterone pellets (2 x 200mg, NV Organon) inserted subcutaneously under local anaesthetic into the anterior abdominal wall on the day of insertion of the etonogestrel implants, and 12 weekly thereafter for the duration of the treatment period, i.e. at 12, 24 and 36 weeks.

During treatment and recovery subjects attended at 4 weekly intervals for medical review, and for semen analysis and venesection. Additional blood samples were drawn

Week	Pre-treatment	Treatment	-3	-1	0	4	8	12	16	20	24	28	32	36	40	44	48
Physical exam	✓							✓			✓			✓			✓
Bioimpedence	✓							✓			✓			✓			✓
Routine bloods ¹	✓							✓			✓			✓			✓
LH/FSH/T																	
SHBG/ENG/ Serum Inhibin B	✓			✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Diurnal T ²	✓			✓	✓	✓	✓	✓									
Epi T (urine)	✓							✓			✓			✓			✓
Semen sample	✓	✓		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Mood/sexual function	✓							✓			✓			✓			✓
Implant insertions		✓															
Implant removals																	✓
T pellets		✓						✓			✓			✓			

Table 4.1: Flow chart of assessments in (pre)treatment phase of subjects receiving 3 Implanon® with 400mg Testosterone pellets 12 weekly
¹Includes haematology, biochemistry, liver function tests and fasting lipids. ² Extra blood samples for T concentrations between 0730-0930 and 1630-1830 at each visit.

pre-treatment and at weeks 4 and 12 between 0730 and 0930 (am samples) and between 1630 and 1830 (pm samples) for testosterone measurement. Subjects were examined at weeks 12, 24, 36, 48 and at final visits, and a morning first-void urine sample was obtained at the same time points for measurement of epitestosterone. Bio-electrical impedance was determined as described (Davies and Preece, 1988; Gregory *et al.*, 1991) using the Holtain Body Composition Analyser (Holtain Limited, Dyfed, Wales) and fat free mass and percentage body fat determined for each subject at screening, 12 weekly thereafter and at 16 weeks of the recovery period. Throughout the study any adverse events were noted at each visit. During the recovery phase, subjects attended at 4 weekly periods for a minimum of 16 weeks up to 24 weeks until semen analysis returned to normal by WHO criteria. Subjects with semen analysis below normal WHO criteria were followed up beyond this period until normal values were attained.

Assays

Blood samples were obtained in fasting subjects (for glucose and lipids) and plasma separated by centrifugation at 4000g for 15 minutes and stored at -20C until hormone assay. Testosterone was measured by radioimmunoassay (RIA) (Corker and Davidson, 1978), and LH, FSH and SHBG by time-resolved immunofluorometric assay. Assay sensitivity was 0.3nmol/L for testosterone, 0.5nmol/L for SHBG, 0.03 IU/l for FSH and 0.15 IU/l for LH. The intra-assay coefficients of variation were less than 10% (testosterone, FSH and LH) and 4% for SHBG. The inter-assay coefficients of variation were 12.4% (testosterone), less than 10% (FSH and LH) and 8.8% for SHBG. Urinary epitestosterone concentrations (aglycone plus free fraction) were determined by gas chromatography-mass spectrometry as described and validated previously (Kicman *et*

al., 1995; Coutts *et al.*, 1997). Between-assay precision was < 8 % for epitestosterone concentrations between 27 and 133 nmol/L, and 13.4 % at 5 nmol/L. Inhibin B was measured in both serum and seminal plasma by methods previously described (Groome *et al.*, 1995; Groome *et al.*, 1996; Anderson *et al.*, 1998) with an assay sensitivity of 7.8 pg/ml. Etonogestrel was measured by in-house RIA by Organon. Samples were analysed for general haematological and biochemical values (including total cholesterol and high-density lipoprotein cholesterol (HDL-C)) by routine autoanalyser at 12 weekly intervals.

Semen analysis

At all assessments semen analysis was carried out using WHO methodology (WHO, 1999). Local normal values for motility are > 27% grade a + b, or > 36% grade a + b + c and normal morphology > 15 %. Azoospermia was confirmed following centrifugation of the whole semen sample. Centrifugation was performed at 3660g for 15 minutes and a sample classified as azoospermic only after a systematic examination of the re-suspended precipitate indicated the complete absence of spermatozoa.

Behavioural assessment

Sexual activity and interest were investigated by means of a structured questionnaire used to quantify sexual activity over the preceding two week period (Anderson *et al.*, 1992). This was carried out before treatment and at 12 weekly intervals thereafter.

Statistical Analysis

Results are presented as mean \pm SEM. Hormone data were log transformed and semen concentrations cube root transformed before analysis by ANOVA for repeated measures. Paired t-tests were used to investigate at what time points significant treatment effects were evident with the exception of behavioural data which was analysed using the Wilcoxon matched pair test for non-parametric testing.

4.3 Results

Subjects, Adverse events and withdrawals

Of the 15 men entering the study, 9 completed 48 weeks treatment, and 4 chose to leave the study after 24 weeks. One man was withdrawn from the study at 24 weeks due to inter-current illness. One man withdrew from the study for personal reasons after 4 weeks treatment, thus data from this individual are not included in the analysis. Adverse events experienced included low mood (3 subjects) and testosterone pellet extrusion (2 subjects) but none resulted in any subject withdrawing from the study. Removal of etonogestrel implants was uncomplicated in all men. Pre-treatment data are presented in Table 4.2.

Sperm concentrations

There was a profound suppression of spermatogenesis during the study (Figure 4.1), and all 14 men became azoospermic eventually. After 16 weeks of treatment, sperm concentration in all subjects was below the threshold of 1×10^6 M/ml with 10/14 subjects (71%) azoospermic (Figure 4.1b). At 24 weeks, 11 men were azoospermic,

Table 4.2 : Pre-treatment values for subjects included in study.
Data presented as mean \pm sem.

Pre-treatment Value	n=15
Age (year)	31.6+/-1.3 (range 18-38)
BMI (kg/m ²)	25.8+/-0.9 (range 20.5-31.9)
LH (IU/litre)	4.0+/-0.4
FSH (IU/litre)	5.3+/-0.8
Testosterone (nmol/litre)	22.1+/-1.7
Sperm concentration (M/ml)	65.9+/-9.8 (median 59.5 range 20.1-137.0)

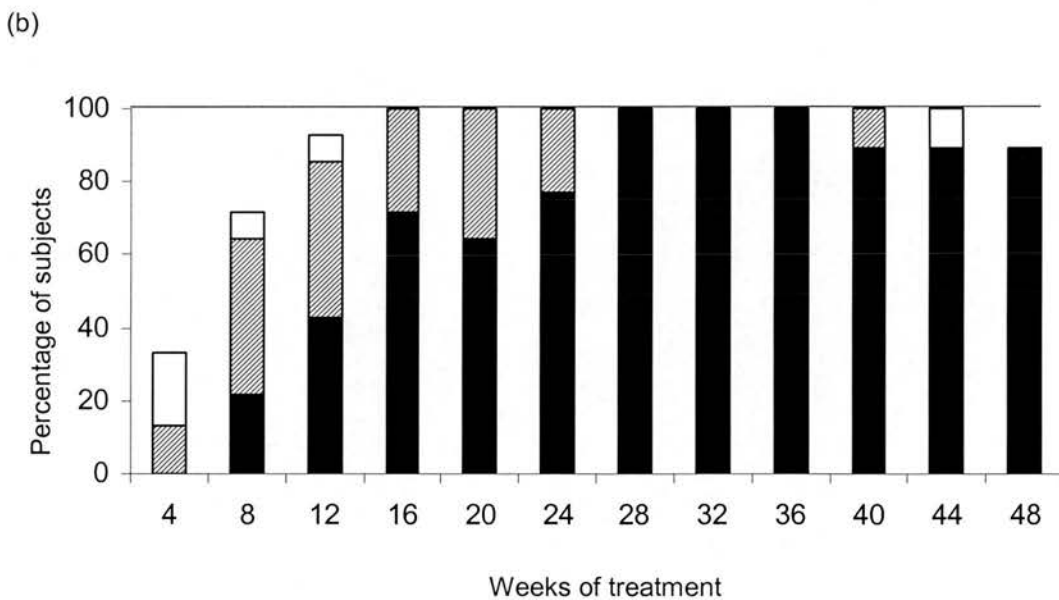
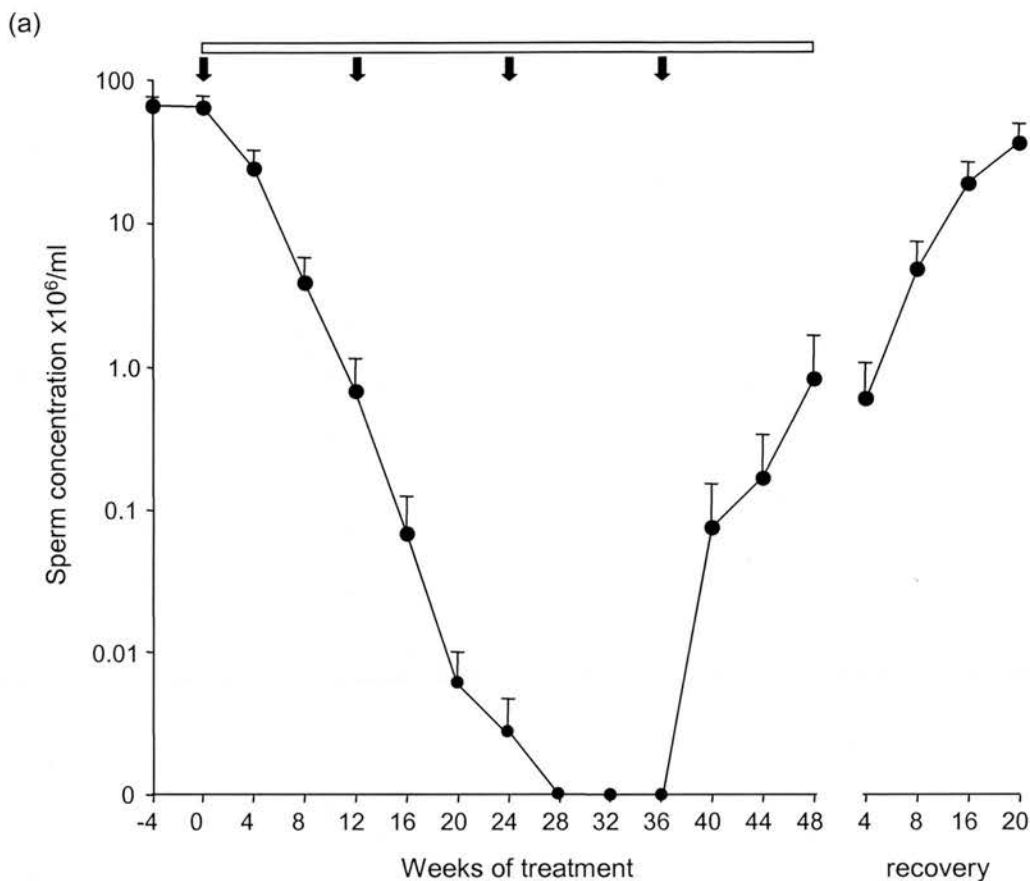


Figure 4.1: (a) Sperm concentrations during etonogestrel/testosterone treatment and recovery. Duration of treatment indicated by bar and time-points of testosterone implant insertion indicated by arrows. Note log scale on ordinate.

Data presented as mean \pm sem, $n=14$ for first 24 weeks, thereafter 9 men continued for 48 weeks.

(b) Percentage of men achieving ■ azoospermia, and concentrations of ▨ less than $1 \times 10^6 \text{ M/ml}$ and □ less than $3 \times 10^6 \text{ M/ml}$ at each time-point during treatment.

and sperm concentrations were $<0.1 \times 10^6/\text{ml}$ in the other three. These three were among the 9 subjects who continued the study for the full 48 weeks, and all were azoospermic at 28 weeks. The range of time to azoospermia was 8-28 weeks, median 16 weeks. Eight men remained azoospermic until the end of the 48 week treatment period. One man showed partial recovery of spermatogenesis, with spermatozoa detectable at week 40 ($0.7 \times 10^6/\text{ml}$) and sperm concentration increasing to $7 \times 10^6/\text{ml}$ at 48 weeks.

During the follow-up phase, 60% of subjects had reached sperm concentrations in the normal range by week 16, and 79% by week 24. Incomplete follow-up data was obtained in the subject who was discontinued from the study due to inter-current illness and in one other man. The remaining two subjects were followed up until normal sperm concentrations were demonstrated at 32 and 48 weeks post implant removal.

Testosterone and etonogestrel concentrations

Serum testosterone concentrations remained within the normal physiological range throughout the treatment period, with fluctuations according to the timing of testosterone pellet re-administration (Figure 4.2a). A gradual decline was observed from pre-treatment values reaching statistical significance at week 4 ($p=0.0006$) with a nadir at week 12. Following re-administration of testosterone at week 12, concentrations rose to levels that were not significantly different from baseline at week 16 with a similar pattern of fluctuation throughout the remainder of the treatment period. During the recovery phase testosterone concentrations rapidly returned to pre-treatment concentrations. Calculated free testosterone concentrations (Vermeulen *et al.*,

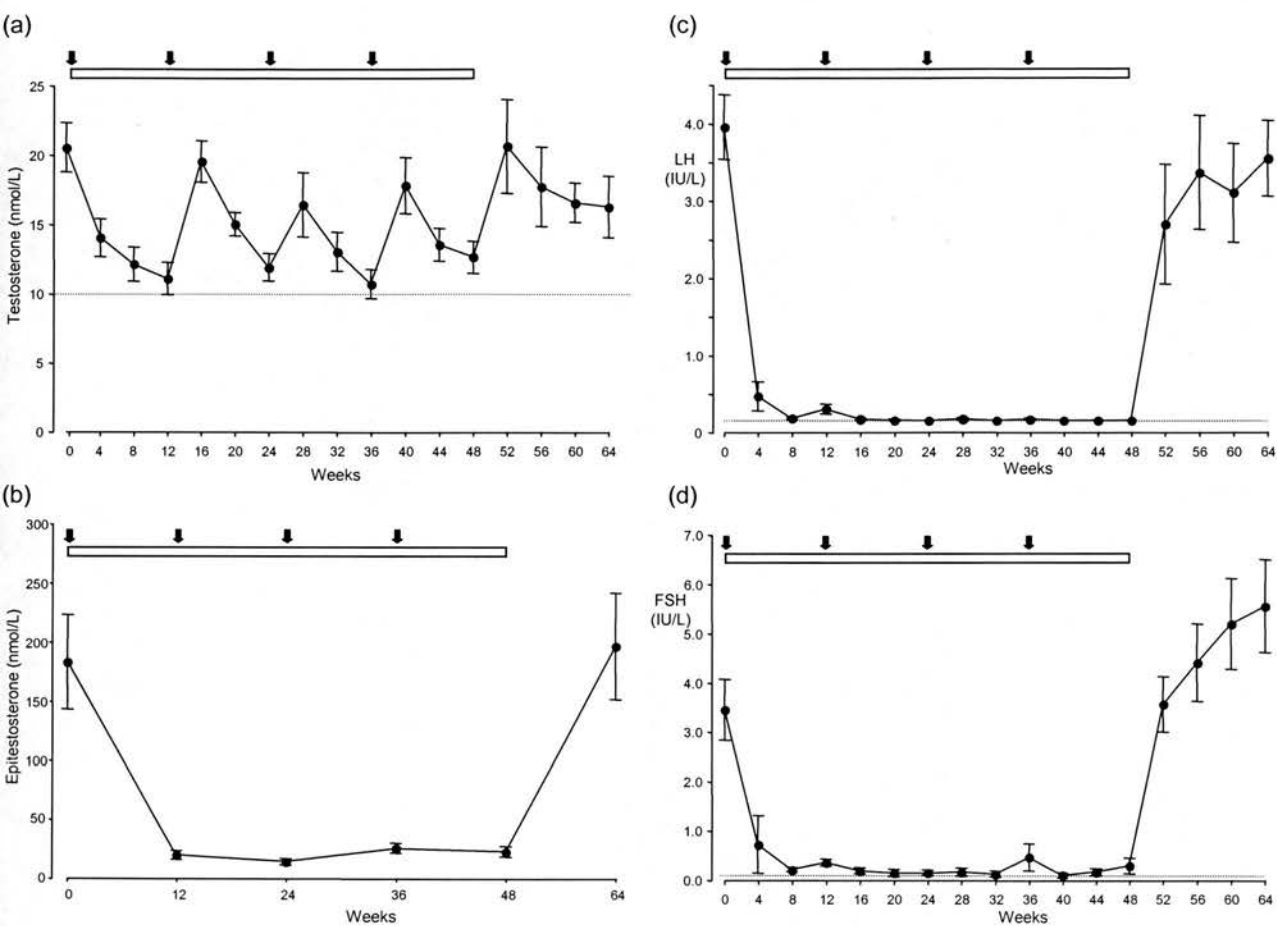


Figure 4.2: (a) Testosterone, (b) Epitestosterone, (c) LH and (d) FSH concentrations during etonogestrel and testosterone treatment. Treatment period is indicated by the bars with time-points of testosterone pellet insertion indicated by arrows. Data presented as mean \pm sem. In Fig 2a, the broken line indicates the lower limit of the normal range. In Figs 2c and d, the lower limit of detection of the assays are indicated by broken lines.

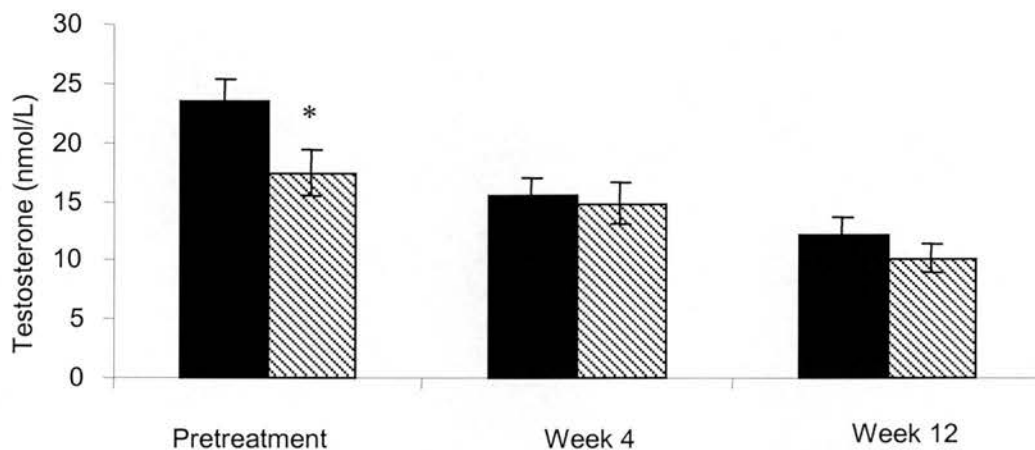


Figure 4.3 Mean ■ am and ▨ pm serum testosterone concentrations (nmol/L) in subjects (n=11) receiving etonogestrel and testosterone treatment pretreatment at after 4 and 12 weeks treatment. Data presented as mean \pm sem. * $p < 0.05$ vs am sample.

1999) showed a similar pattern, with nadir concentrations significantly lower than pre-treatment ($p < 0.01$, Table 4.4) and returning to pre-treatment levels during the recovery phase. During the treatment phase free testosterone concentrations showed a gradual rise from week 12 (0.30 ± 0.03 nmol/L) to week 48 (0.39 ± 0.03 nmol/L) which was not statistically significant.

Urinary epitestosterone concentrations were suppressed by week 12 ($p = 0.001$) to approximately 10% of pre-treatment concentrations (Figure 4.2b). Epitestosterone concentrations remained consistently suppressed throughout treatment without significant change, and returning to pre-treatment concentrations by 12 weeks of recovery.

A diurnal variation in serum testosterone concentrations was observed pre-treatment (Figure 4.3), concentrations in the morning being an average of 35% higher than in the early evening ($p = 0.002$). After 4 weeks of treatment this was lost, with no significant differences between morning and evening concentrations. Concentrations at both times of day at 4 weeks however were not significantly different from pre-treatment early evening concentrations. At 12 weeks of treatment, mean testosterone concentrations were low being immediately prior to re-administration of the testosterone pellets, but were again similar in the morning and evening. Comparison of the diurnal variation in testosterone concentrations between pre-treatment and 12 weeks showed a significant difference ($P < 0.05$).

Other reproductive hormones

Treatment with etonogestrel and testosterone resulted in profound suppression of both LH and FSH ($p < 0.0001$ vs pre-treatment from week 4 onwards). Some fluctuation in suppression was evident at 12 and 36 weeks (FSH) and 12 weeks (LH), at the times of trough testosterone concentrations (Figure 4.2c and d). This reached statistical significance at 16 weeks for FSH ($p = 0.01$ vs 12 weeks) following testosterone implant re-administration at week 12. During the later weeks of the study, LH was consistently suppressed to undetectable concentrations in all men at 24 weeks of treatment and for the rest of the treatment period in all men who continued to 48 weeks. Suppression of FSH was more variable, being detectable in up to 2/3rds of subjects at time points of trough testosterone concentrations. More consistent partial recovery of FSH concentrations was seen in three men during the final 8 weeks of the study, particularly in the one individual who showed some restoration of spermatogenesis. In this individual FSH concentration during the second half of the treatment period was undetectable only at week 40, with a mean concentration between weeks 28 and 48 of 0.5 IU/L. Two further individuals with partial escape of FSH suppression (mean concentrations between weeks 28-48 of 0.1IU/L and 0.8IU/l) maintained azoospermia. Both gonadotrophins rapidly recovered following treatment. There was a progressive rise in FSH from weeks 4 to 16 of the recovery phase, at which time FSH concentrations were significantly higher than pre-treatment ($p = 0.02$).

Serum inhibin B concentrations showed a gradual decline over the course of treatment, continuing to week 48 ($p < 0.001$; Figure 4.4). This reached statistical significance from week 4 of treatment onwards ($p = 0.047$). By week 16 of the recovery phase, serum

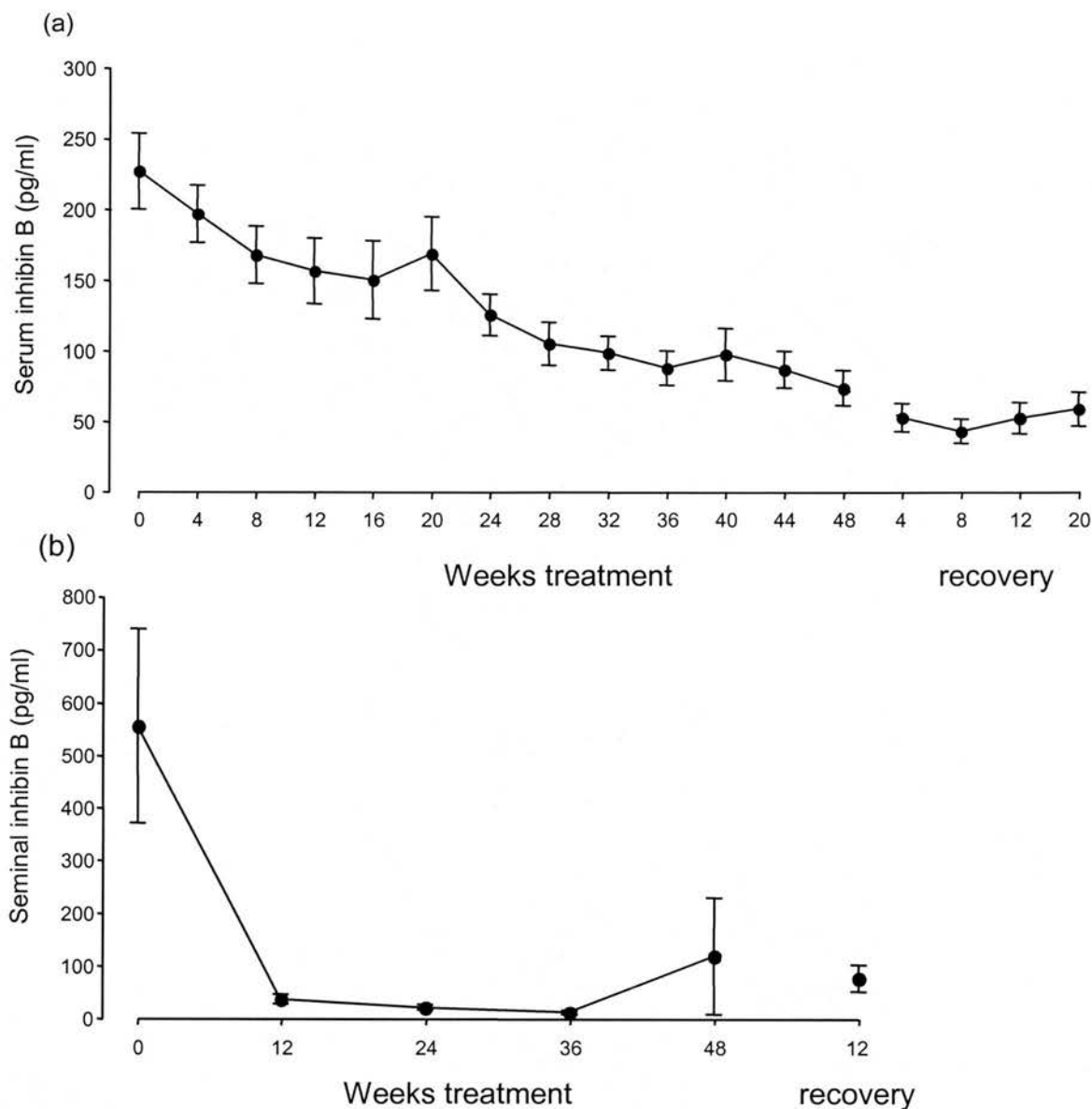


Figure 4.4: (a) Serum inhibin B and (b) seminal plasma inhibin B concentrations during etonogestrel and testosterone treatment. Treatment period indicated by bar with time-points of testosterone implant insertion by arrows. Data presented as mean \pm sem. The large error bar at 48 weeks (Fig 4b) reflects partial recovery in one individual: seminal plasma inhibin B was undetectable in all other samples at that time.

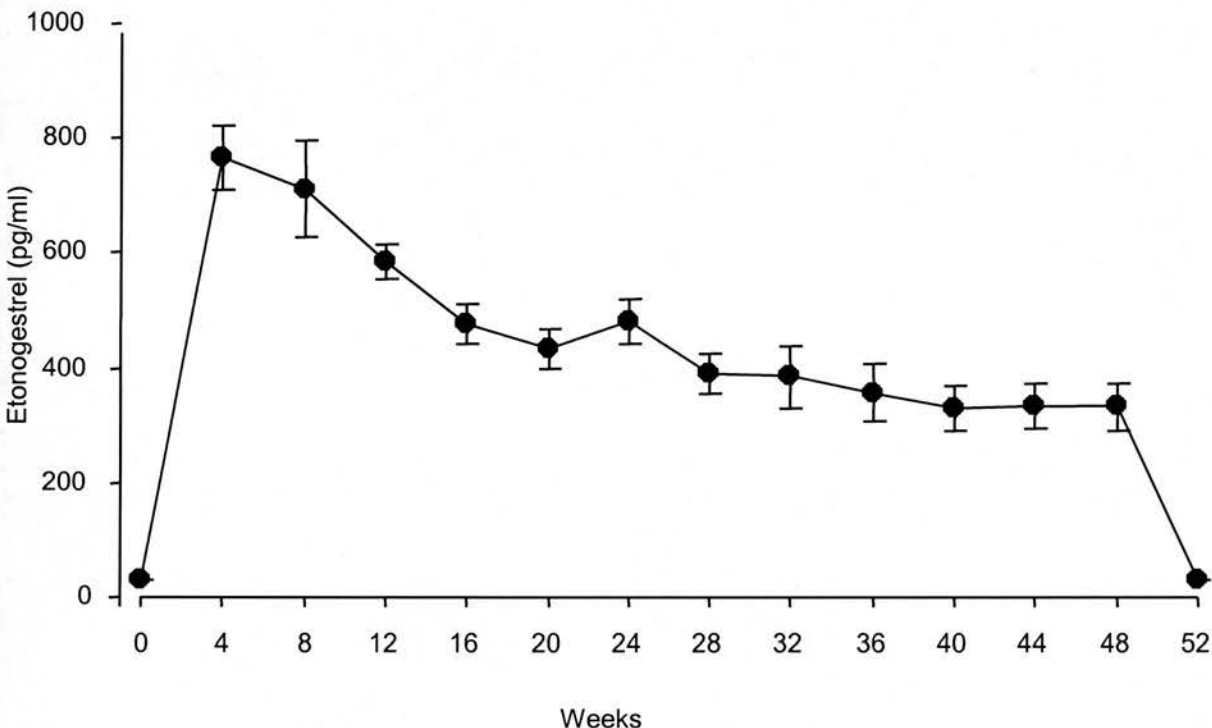


Figure 4.5: Etonogestrel concentrations during etonogestrel/testosterone treatment (0-48 weeks) and 4 weeks after removal of etonogestrel implants, at which time it was undetectable in all men. Data presented as mean \pm sem.

inhibin B levels showed only limited evidence of recovery, remaining significantly lower than pre-treatment ($p < 0.001$).

Seminal plasma inhibin B concentrations were profoundly suppressed during treatment ($p = 0.02$ pre-treatment vs week 12). Seminal plasma inhibin B was undetectable in 8 of 13 subjects by week 24. In the latter 24 weeks of the study it was undetectable in all subjects except the individual who demonstrated recovery of spermatogenesis. This subject showed an increase in seminal plasma inhibin B at 36 weeks (having been at the limit of detection at week 24). This thus preceded detectable spermatogenic recovery, as at that time the subject was azoospermic but had a sperm concentration of $0.7 \times 10^6/\text{ml}$ 4 weeks later.

SHBG showed a gradual decline over the treatment period (Table 4.4). This reached statistical significance by week 4 ($p = 0.0002$) and continued to week 48. During recovery, SHBG returned to pre-treatment concentrations.

Etonogestrel

Serum etonogestrel concentrations were highest 4 weeks after implant insertion with a mean concentration of $765 \pm 57 \text{ pg/ml}$. Etonogestrel concentrations showed a gradual decline thereafter (Figure 4.5), being 63% of peak levels at 24 weeks and 43% at week 48. Etonogestrel was undetectable in all subjects 4 weeks after implant removal.

Lipids and Haematology

Table 4.4: Haematological, lipid, SHBG and free testosterone concentrations pre-treatment and during treatment at indicated time-points and after 16 weeks recovery. Data presented as mean±SEM.

	Pretreatment	12wk	24wk	36wk	48wk	Recovery
Haemoglobin (g/l)	152±1.7	152±2.6	154±1.5	154±2.4	155±2.1*	157±2.1*
Haematocrit	0.45±0.01	0.44±0.01	0.45±0.01	0.45±0.01	0.45±0.01	0.46±0.01
Cholesterol (mmol/L) ^a	5.3±0.4	5.0±0.4	4.6±0.3*	4.9±0.3	4.5±0.3	5.3±0.7
HDL-C (mmol/L)	1.2±0.1	1.1±0.1	1.1±0.1	1.1±0.1	1.1±0.1	1.3±0.2
LDL-C (mmol/L)	3.7±0.4	3.5±0.4	3.2±0.3	3.5±0.3	3.3±0.2	3.6±0.4
Triglycerides (nmol/L) ^b	2.1±0.3	1.7±0.4	1.6±0.2*	1.5±0.3	1.2±0.2*	1.9±0.4
Free testosterone (nmol/L) ^c	0.52±0.05	0.30±0.03*	0.34±0.02*	0.33±0.03*	0.39±0.03	0.44±0.09
SHBG (nmol/L) ^d	25.6±2.7	18.2±1.6*	15.5±1.4*	12.2±1.9*	13.2±2.2*	22.2±2.9

^a Cholesterol concentrations were significantly lower at 24 weeks of treatment (p=0.006).

^b Triglyceride concentration was significantly lowered at wk24 (p=0.05) and wk48 (p=0.02) of treatment.

^c Free testosterone concentrations were significantly lower from wk12 (p=0.003) until wk 36

^d SHBG concentrations were significantly lower from wk 12 (p=0.001) and remained significantly decreased until recovery

Further significant (p<0.05) treatment changes following ANOVA indicated by *.

Cholesterol concentrations showed a gradual fall during etonogestrel and testosterone treatment, statistically significant at week 24 ($p=0.006$) and returning to baseline during recovery (Table 4.4). Similarly, there was a decline in triglycerides, reaching significance at week 24 ($p=0.05$) and week 48 ($p=0.02$). There was no significant change in HDL-C levels with a small (10%) non-significant decline in LDL-C during the treatment period. There were no significant changes in other biochemical variables during the study period.

A small rise in haemoglobin concentrations was evident at week 48 ($p=0.003$), which remained elevated during the recovery period. Haematocrit remained unchanged.

Body Composition

There were no significant changes in body weight during the treatment or recovery periods. Likewise, body composition analysis showed no changes in fat free mass or percentage body fat (Table 4.3).

Sexual Behaviour

There was a slight increase in sexual activity (recorded as the sum of number of acts of sexual intercourse and masturbation over the preceding 2 weeks) at week 12 of treatment ($p=0.04$) (Table 4.5). No changes in sexual activity at other time points were observed during the study.

Table 4.3: Body composition data (Weight, total body water, fat free mass, fat mass and % body fat) pre-treatment, 12 weekly during treatment and after 16 weeks of follow up. Data presented as mean±SEM.
No significant changes indicated.

	Pretreatment	12wk	24wk	36wk	48wk	Recovery
Weight (kg)	82.4±3.4	81.6±3.5	81.3±3.2	82.4±2.8	83.7±2.9	80.9±3.5
Total Body Water (l)	47.2±1.6	45.5±1.8	45.9±1.7	47.0±1.9	46.5±1.4	46.4±1.7
Fat Free Mass (kg)	64.6±2.2	62.3±2.4	64.4±2.6	64.4±2.6	63.7±1.9	63.6±2.4
Fat Mass (kg)	17.7±2.4	18.0±2.7	18.3±1.9	18.0±2.6	19.9±2.9	16.0±2.4
% Body Fat	20.7±2.3	21.5±2.6	22.1±1.9	21.6±2.8	23.3±2.9	19.4±2.5

Table 4.5: Sexual behaviour pre-treatment, 12 weekly during etonogestrel/testosterone treatment and after 16 weeks of follow-up. Data presented as mean±SEM.

	Pretreatment	12wk	24wk	36wk	48wk	Recovery
Sexual Behaviour	6.2±1.2	7.4±1.7*	6.5±1.8	5.3±0.8	5.3±1.5	4.8±0.9

Sexual activity was assessed as the sum of acts of masturbation and intercourse during the preceding 2 weeks.
* indicates a significant increase at week 12 of treatment (p=0.04).

4.4 Discussion

One of the major hurdles in the development of a hormonal male contraceptive is the need for sufficient and universal suppression of spermatogenesis. Caucasian populations have shown heterogeneous responses to both testosterone alone and in combination with progestogens (World Health Organisation Task Force on Methods for the Regulation of Male Fertility, 1990; Anderson and Baird, 2002) although the addition of a progestogen has generally increased the proportion of men achieving azoospermia. Previously very high rates of azoospermia have been demonstrated using oral desogestrel as the progestogen (Wu *et al.*, 1999; Anawalt *et al.*, 2000; Kinniburgh *et al.*, 2001; Kinniburgh *et al.*, 2002). Administration of an implant preparation of etonogestrel, the active metabolite of desogestrel, also resulted in effective suppression of spermatogenesis (Anderson *et al.*, 2002a). In the present study both the dose-response relationship, and the duration of action of etonogestrel implants when administered with a depot testosterone preparation have been further explored.

The present data demonstrate profound suppression of spermatogenesis with the combination of three etonogestrel implants and depot testosterone pellets, with all subjects achieving azoospermia. This compares favourably with previous data using one (64% azoospermia) and two implants (75% azoospermia) over a 24 week period (Anderson *et al.*, 2002a) and is similar to that achieved with an oral dose of 300µg desogestrel with the same regimen of testosterone administration (Kinniburgh *et al.*, 2002). The onset of suppression was rapid, with all subjects having sperm concentrations of less than 1×10^6 M/ml by week 16 of treatment. However the time taken to reach azoospermia was considerably more variable, with 3 men maintaining

very low but detectable numbers of sperm in the ejaculate to up to 28 weeks. Similar data are evident from the recent Australian efficacy study (Turner *et al.*, 2003) despite the very rapid suppression achieved by that combination of testosterone pellets and depot medroxyprogesterone acetate, whereby 94% of men achieved a sperm concentration of $<1 \times 10^6$ M/ml within 3 months. This may have significant implications for the practicality of the method, depending on the threshold required for acceptable contraceptive efficacy (Nieschlag, 2002).

Mean serum etonogestrel concentrations of approximately 1200pg/ml and 500-800pg/ml were reported for 300µg and 150µg oral desogestrel respectively (Wu *et al.*, 1999; Anawalt *et al.*, 2000). In the present study, the serum etonogestrel concentration at 12 weeks was approximately 600pg/ml. Thus the suppressive effect of this preparation is similar to that of 300µg desogestrel per day, whereas the dose is similar to 150µg/day. Dose-sparing is also evident with this preparation of testosterone (Handelsman *et al.*, 1992), which maintains relatively stable serum concentrations and particularly avoids the supraphysiological peaks observed with esters such as testosterone enanthate (World Health Organisation Task Force on Methods for the Regulation of Male Fertility, 1990). The dose of testosterone administered here has no significant suppressive effect on spermatogenesis when given alone (Handelsman *et al.*, 2000), and in combination with a progestogen may be the minimum effective dose. The advantageous features of this testosterone preparation will contribute to minimising the intratesticular testosterone concentration which is recognised to be of importance in maximising spermatogenic suppression (Meriggiola *et al.*, 2002; Zhang *et al.*, 2003).

Non-reproductive adverse effects of weight gain and changes in plasma lipoproteins were reduced compared to studies investigating oral desogestrel (Wu *et al.*, 1999; Anawalt *et al.*, 2000; Kinniburgh *et al.*, 2002) and to combinations of injectable testosterone with other gestogens (Bebb *et al.*, 1996; Anawalt *et al.*, 1999; Kamischke *et al.*, 2001). The reduced non-reproductive impact is also exemplified by the lack of change in the body composition components during the study. Physiological doses of testosterone increase fat free mass in hypogonadal and old men (Bhasin *et al.*, 1997; Bhasin *et al.*, 1998; Snyder *et al.*, 1999; Snyder *et al.*, 2000), and supraphysiological doses increase fat free mass in normal men (Young *et al.*, 1993; Bhasin *et al.*, 1996). In the setting of male contraception, an additive effect of testosterone enanthate and levonorgestrel increasing lean mass has been demonstrated (Herbst *et al.*, 2003). In this study the absence of effect on body composition is likely to reflect the fact that the dose of testosterone is within the physiological range.

The diurnal variation of testosterone concentrations in adult men has been well characterised (Faiman and Winter, 1971; Bremner *et al.*, 1983) if not understood. The dose of testosterone which is physiological is usually considered to be that which reproduces the peak concentration observed in men during the morning (World Health Organisation *et al.*, 1992). This may result in the administration of a higher dose than that required for physiological replacement. In this study a preliminary investigation of diurnal variation in serum testosterone before and during testosterone/progestogen administration was carried out, which we hypothesised would not be detectable during exogenous steroid administration if it was primarily due to variation in testosterone production rather than metabolism (Southren *et al.*, 1967). The data confirmed that the

diurnal variation of testosterone was lost during treatment, at both 4 and 12 weeks. Testosterone concentrations at 4 weeks were similar to pre-treatment evening samples and although testosterone concentrations at 12 weeks treatment were lower than both pre-treatment morning and evening samples, they were taken at the nadir, immediately prior to re-administration and thus are not representative of 'average' testosterone concentrations over the treatment period. This regimen may therefore more closely replace the trough of physiological diurnal production rather than the peak, and it is likely that this will have contributed to the lack of non-reproductive changes observed here.

Gonadotrophin secretion was profoundly suppressed during treatment. This was particularly marked with LH, which was undetectable in all subjects by week 24 and remained so for the duration of treatment. Suppression of FSH was more variable, remaining detectable in a proportion of subjects. Nonetheless, suppression of FSH was greater than with 1 or 2 implants (Anderson *et al.*, 2002a). The 12 week testosterone administration regimen also appears more effective at preventing FSH escape than the same total dose administered at 24 week intervals (Turner *et al.*, 2003). With assays of even higher sensitivity (limit of detection 0.01IU/L), it appears that circulating FSH is detectable in most men using similar regimens despite achievement of azoospermia (McLachlan *et al.*, 2002b). Desogestrel and other progestogens may result in increased spermatogenic suppression than achieved by comparable gonadotrophin suppression using testosterone alone (McLachlan *et al.*, 2002b), consistent with direct testicular effects on steroidogenesis (Satyaswaroop and Gurpide, 1978; El-Hefwany and Huhtaniemi, 1998; El-Hefwany *et al.*, 2000) or androgen metabolism (Mauvais-Jarvis *et*

al., 1974). In the present study, FSH was incompletely suppressed during weeks 24-48 in three subjects, only one of whom showed spermatogenic recovery. While adequate suppression of FSH is clearly necessary for achievement of azoospermia (Narula *et al.*, 2001; Weinbauer *et al.*, 2001) it appears that there is no clear threshold below which azoospermia can be confidently predicted, and that FSH suppression is only one of a number of potential determining factors for incomplete suppression or escape of spermatogenesis. Consistent with the reproducible suppression of LH, urinary excretion of epitestosterone fell to approximately 10% of pre-treatment values and remained at that level for the duration of treatment. Epitestosterone (17 α -hydroxyandrost-4-en-3-one) is a natural epimer of testosterone secreted predominantly by the testis (Kicman *et al.*, 1999) which therefore provides a measure of endogenous testicular secretion. Epitestosterone excretion during the present treatment regimen was similar to that previously reported during oral desogestrel/testosterone treatment of normal men (Kinniburgh *et al.* 2002), and is significantly higher than in hypogonadal men (Kicman *et al.*, 1999). Direct measurement of intratesticular testosterone also indicates low ongoing testosterone production despite near complete LH suppression (McLachlan *et al.* 2002) but there are no data directly comparing intratesticular testosterone concentrations with epitestosterone excretion. Comparison of epitestosterone excretion between different regimens might allow non-invasive exploration of the relationship between ongoing testicular steroidogenesis and spermatogenesis during profound gonadotrophin suppression.

The concentration of inhibin B provides an overall measure of Sertoli cell number and function including spermatogenesis (Anderson and Sharpe, 2000). While it would be

expected that effective hormonal contraceptive regimens would result in significant falls in inhibin B concentrations, this has not always proved to be the case. Some studies have reported a fall in inhibin (Anawalt *et al.*, 1996; Anderson *et al.*, 1997b; Zhengwei *et al.*, 1998), but others have not (Büchter *et al.*, 1999; Martin *et al.*, 2000b; Kinniburgh *et al.*, 2002). Significant changes have not been found in previous studies with both oral desogestrel and etonogestrel implants despite the high prevalence of azoospermia. It is likely that changes in circulating inhibin B require profound regression of spermatogenesis more consistently throughout the testis than is achieved with some regimens (Anderson and Sharpe, 2000). This is supported by testis biopsy data showing variable degrees of spermatogenic regression between nearby seminiferous tubules despite induction of azoospermia (McLachlan *et al.*, 2002b). The fall in inhibin B observed in the present study may therefore reflect a greater consistency of suppression than is reflected purely by the prevalence of azoospermia. This is supported by the striking fall in the concentration of inhibin B in the ejaculate. It has also been demonstrated that seminal plasma inhibin B is of testicular origin (Anderson *et al.*, 1998), and fell to undetectable concentrations in 10/12 men receiving a highly efficacious dose of oral desogestrel resulting in universal azoospermia (Kinniburgh *et al.*, 2002). More variable falls were found in previous studies with one or two etonogestrel implants (Anderson *et al.*, 2002a). In this study, with three etonogestrel implants these previous findings are confirmed and it seems that changes in seminal inhibin B are a sensitive window into the seminiferous epithelium, as seminal inhibin B was profoundly suppressed in all men to a median of <10pg/ml at 24 weeks treatment. This is supported by observations in the individual who demonstrated recovery of

spermatogenesis during treatment, as the appearance of sperm in the ejaculate was intriguingly preceded by a partial recovery of seminal plasma inhibin B.

Other long-term approaches to male hormonal contraception have involved implants and depot injections. Levonorgestrel has also been administered in implant formulation (Norplant II®) with azoospermia achieved in 35% of subjects when given with transdermal testosterone patches and 93% of subjects in combination with weekly testosterone enanthate (Gao *et al.*, 1999; Gaw Gonzalo *et al.*, 2002). The combination with testosterone implants or long-acting injectable preparations has yet to be investigated. 7 α -Methyl-19-nortestosterone (MENT), a synthetic androgen more potent than testosterone and resistant to 5 α -reduction (Sundaram *et al.*, 1993), has also recently been developed as an implant and a potential long-acting male contraceptive. However even when up to 4 implants were used (a dose which resulted in significant effects related to excess androgenicity) approximately 30% of men still had significant numbers of sperm in the ejaculate (von Eckardstein *et al.*, 2003) consistent with the limitations of an androgen-only approach in Caucasian men. Thus, of implant approaches to date the combination presented in this study exhibits higher levels of spermatogenic suppression with a more favourable side-effect profile than any of the others. This beneficial therapeutic ratio is likely to reflect the pharmacokinetics of both the testosterone and progestogen preparations. Other promising long-term approaches include long-acting injectable testosterone undecanoate alone achieving high levels of oligozoospermia and azoospermia among Chinese men (Gu *et al.*, 2003), the depot injectable combination of norethisterone enanthate and testosterone undecanoate (Kamischke *et al.*, 2002) and DMPA with testosterone pellets (Turner *et al.*, 2003).

In conclusion, the results in this study demonstrate that administration of etonogestrel implants at an appropriate dose together with a long-acting testosterone preparation induces profound and consistent suppression of spermatogenesis that can be maintained for a period of 1 year. Whether this time period could be extended remains to be investigated. The maintenance of testosterone concentrations within the eugonadal range and the dose-sparing effects of the delivery methods involving constant release may contribute to the lack of non-reproductive effects. This approach exemplifies the basis for an acceptable, long-acting, and reversible male hormonal contraceptive.

CHAPTER 5

DEMONSTRATION OF PROGESTERONE RECEPTOR-MEDIATED GONADOTROPHIN SUPPRESSION IN THE HUMAN MALE

5.1 Introduction

It has been demonstrated in the preceding studies in this thesis that one of the most promising approaches to hormonal male contraception involves the administration of testosterone with a gestogen, acting predominantly by suppression of gonadotrophin secretion (Meriggiola and Bremner, 1997; Nieschlag *et al.*, 2003). The addition of the second agent allows lower doses of each steroid to be given thus avoiding side effects from supraphysiological doses of testosterone while maintaining effective spermatogenic suppression. Although having additive effects on the suppression of gonadotrophins (Wu *et al.*, 1999) the underlying mechanisms and the physiological importance of progestogens in suppressing gonadotrophin secretion in the male remain largely unknown. The synthetic gestogens, in particular the 19-norgestogens, have affinity for both androgen and progesterone receptors but the relative contribution of these two components in suppressing gonadotrophins is unclear.

In animal models, investigation of the effect of progesterone on gonadotrophin secretion in the male has given conflicting results. Following a large single injection of progesterone in adult rams plasma LH levels were suppressed (Bolt, 1971). However, in other studies using infusions (Edgerton and Baile, 1977) or implants (Echternkamp and Lunstra, 1984; Van Lier *et al.*, 1999) no effect was reported. Recently progesterone administration was found to suppress LH in castrate rams when given with testosterone

(Turner *et al.*, 2001). The male progesterone receptor knock-out mouse demonstrates elevated circulating LH concentrations (Schneider *et al.*, 1999) suggesting a functional role for the progesterone receptor in the control of LH secretion.

Desogestrel is a synthetic third generation 19-nortestosterone derivative. The active metabolite, etonogestrel, demonstrates relatively high progesterone receptor selectivity in comparison to other synthetic gestogens such as gestodene and levonorgestrel (Phillips *et al.*, 1990). It has been a valuable component of the female combined oral contraceptive pill for many years, demonstrating desirable progestational activity with low androgenic effect. Recent studies of desogestrel in combination with testosterone esters as a hormonal contraceptive in men have had promising results, with azoospermia induced in 90 to 100% of subjects (Wu *et al.*, 1999; Anawalt *et al.*, 2000; Martin *et al.*, 2000b; Kinniburgh *et al.*, 2002).

This study aimed to compare the effects of desogestrel with naturally occurring progesterone on LH and FSH secretion in healthy men. To date there have been no studies in men comparing the effect of progesterone with desogestrel on the secretion of gonadotrophins. Progesterone, the natural steroid, has very low affinity for the androgen receptor, whereas desogestrel, although comparatively more potent and of greater selectivity than most of the synthetic gestogens, binds to both the progesterone and androgen receptors. Therefore, the hypothesis was that if progesterone and desogestrel suppressed gonadotrophins to a similar extent it was unlikely that the suppressive effects of synthetic gestogens in the male are solely due to their androgenic properties.

5.2 Subjects and methods

Subjects

Twenty normal men, aged 18-38, were recruited to participate in this study. A power calculation was not performed in this study. Study size was based on previous experience of the number of subjects required to detect statistically significant and clinically meaningful changes in the primary end-points. They had a normal medical history and examination and haematological and biochemical screening obtained within 2 weeks of the start of the study. They were receiving no medication. Serum testosterone, follicle stimulating hormone, luteinising hormone and inhibin B concentrations, determined between the hours of 0800 and 1100, revealed no clinically abnormal findings.

Experimental protocol

The study protocol was reviewed by the Lothian Research Ethics Committee and informed consent obtained. The study design was a single-centre open parallel-group study. Subjects were admitted to the clinical research area for a control period of frequent blood sampling, carried out at 15 minute intervals via an indwelling catheter from 0800 until 2000 hours. Two hours before the end of sampling, subjects were administered 100µg GnRH i.v. Subjects were semi-ambulant throughout and did not fast. Subjects were then randomised by blocks of consecutive subjects to receive either desogestrel 300µg p.o. daily or progesterone 50mg i.m. daily for 7 days from the day following the first frequent sampling with the final dose administered on the morning of the second frequent blood sampling day. All samples were assayed for LH, and hourly

samples for testosterone, FSH and inhibin B. Two weeks after the second frequent sampling period subjects attended for a post-treatment visit noting any adverse events.

Drugs

Desogestrel (NV Organon, Oss, The Netherlands) was taken at a dose of 300 μ g (2x150 μ g tablets) p.o. daily. Progesterone (Ferring Pharmaceuticals Ltd., Langley, Berkshire) 50mg in 1ml intramuscularly was administered daily by the investigator. GnRH (Fertinal, Hoechst Marion Rousell Ltd., Tokyo, Japan) was administered at a dose of 100 μ g intravenously to each subject after 10 hours frequent blood sampling which was then continued for a further 2 hours.

Hormone Assays

Blood was immediately centrifuged, plasma separated and stored at -20° C until assayed. All samples were assayed for LH, and hourly samples for FSH, testosterone and inhibin B. Testosterone was measured by radioimmunoassay (Corker and Davidson, 1978) with a detection limit of 0.2nmol/L and intra-assay variability of 8.9%. Plasma LH and FSH were measured by time-resolved immunofluorometric (in-house) assay and by highly sensitive immunoradiometric assay respectively (NETRIA, St Bartholomew's Hospital, London, UK): assay sensitivity was 0.15 IU/L (LH) and 0.1 IU/L (FSH) and coefficients of variation 9% (LH) and 8% (FSH). Inhibin B was measured as previously described (Groome *et al.*, 1996). The detection limit of the assay was 15pg/ml with an intra-assay coefficient of variation of 10.5%. Progesterone was measured using DPC Coat-A-Count Radioimmunoassay kit (DPC Biermann GmbH Diagnostika, Bad Nauheim, Germany), with a sensitivity of 0.06nmol/L.

Statistical Analysis

Mean hormone levels were calculated over 10 hours of frequent blood sampling prior to the administration of GnRH. Pulses were identified by a modified version of that described by Santen and Bardin (Santen and Bardin, 1973) defining a pulse as an increment of greater than 20% sustained over 2 time points from the mean of the 2 values at the pulse nadir. Mean pulse frequency was then calculated over 10 hours prior to the administration of GnRH and pulse amplitude from the peak to nadir difference (IU/L). The response to GnRH was calculated from the mean hormone values following the administration of GnRH over the final 2 hours of frequent blood sampling. An alternative method, calculating the absolute difference from the peak and nadir concentrations, gave similar results. Results are presented as mean \pm SEM. Hormonal data were analysed using Students t-tests, paired when appropriate, on log transformed data to correct heterogeneity of variance.

5.3 Results

Baseline Characteristics

There were no significant differences between both groups with regards to age, body mass index, and pre-treatment testosterone and LH concentrations. However pre-treatment FSH concentrations were significantly higher in the desogestrel group, although no values were outwith the normal range ($<7\text{IU/L}$) (table 5.1). Serum progesterone concentrations at 0.75hrs and 9.25hrs of frequent blood sampling following 7 days of progesterone administration were $113.2 \pm 14.4 \text{ nmol/L}$ and $116.6 \pm 8.2 \text{ nmol/L}$ respectively.

Table 5.1: Baseline characteristics of subjects

Parameter	Progesterone	Desogestrel
Age (years)	24.2±1.5	25.4±1.5
BMI (kg/m ²)	23.8±0.9	24.3±1.0
Testosterone (nmol/L)	19.5±2.6	21.4±2.6
LH(IU/L)	4.5±0.9	4.4±0.9
FSH (IU/L)	3.1±0.2	4.3±1.0*

Data presented as mean±SEM, *p<0.05 versus progesterone group

Table 5.2: Mean hormone concentrations (pre-GnRH administration)

Parameter	Progesterone		Desogestrel	
	Control	Treatment	Control	Treatment
Testosterone (nmol/L)	21.68±1.94	8.75±0.68*	21.58±2.87	8.03±2.09*
LH (IU/L)	2.92±0.71	1.57±0.32*	2.58±0.39	1.42±0.36*
FSH (IU/L)	2.14±0.22	1.09±0.15*	2.88±0.34	1.48±0.20*
Inhibin (IU/L)	211.8±36.6	186.9±19.7	219.6±35.0	229.7±30.4

Data presented as mean±SEM, *p<0.005 versus control. Means are average of all data over 10 hrs pre-GnRH administration.

Mean hormone concentrations

Both desogestrel and progesterone treatment resulted in significant falls in the mean concentrations of LH and FSH ($p<0.005$, Table 5.2) and of testosterone ($p<0.005$). There was no significant change in serum inhibin B with either treatment. However a greater sample size may have detected a change in inhibin B concentrations.

Pulsatile LH secretion

LH pulse amplitude was similar in the two groups pre-treatment (figure 5.1), although the mean number of pre-treatment pulses in 10 hours was lower in the desogestrel group than in the progesterone group ($p=0.03$). There was a significant decline in pulse amplitude with both treatments, from 2.4 ± 0.5 to 1.6 ± 0.4 IU/L ($p<0.05$) in the progesterone-treated group and from 1.9 ± 0.2 to 1.3 ± 0.3 IU/L ($p<0.05$) in the desogestrel-treatment group (figure 5.1). There was also a significant decline in the frequency of LH pulses following progesterone treatment, from 4.7 ± 0.4 to 3.8 ± 0.4 per 10 hours ($p<0.005$) (figures 5.1 and 5.2a). Desogestrel treatment resulted in a non-significant decrease in LH pulse frequency, from 3.5 ± 0.2 to 3.0 ± 0.3 per 10 hrs ($p=0.3$) (figures 5.1b and 5.2).

Gonadotrophin response to GnRH

All men showed brisk increases in both LH and FSH secretion in response to GnRH administration. There was a significant attenuation of the response to GnRH in the progesterone-treated group. Mean post-GnRH LH concentration fell from 16.3 ± 4.3 to 10.5 ± 2.6 IU/L ($p<0.0005$), and FSH fell from 3.6 ± 0.3 to 2.0 ± 0.2 IU/L ($p<0.005$) (fig 5.3). In the desogestrel-treated group there was a significant fall in FSH from 4.5 ± 0.5

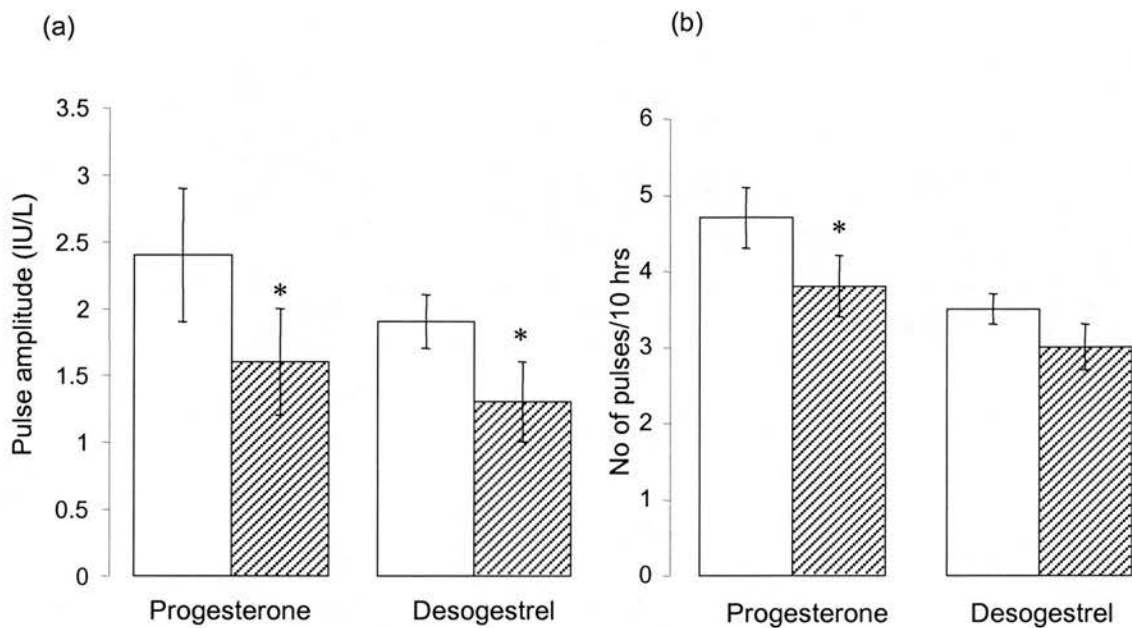
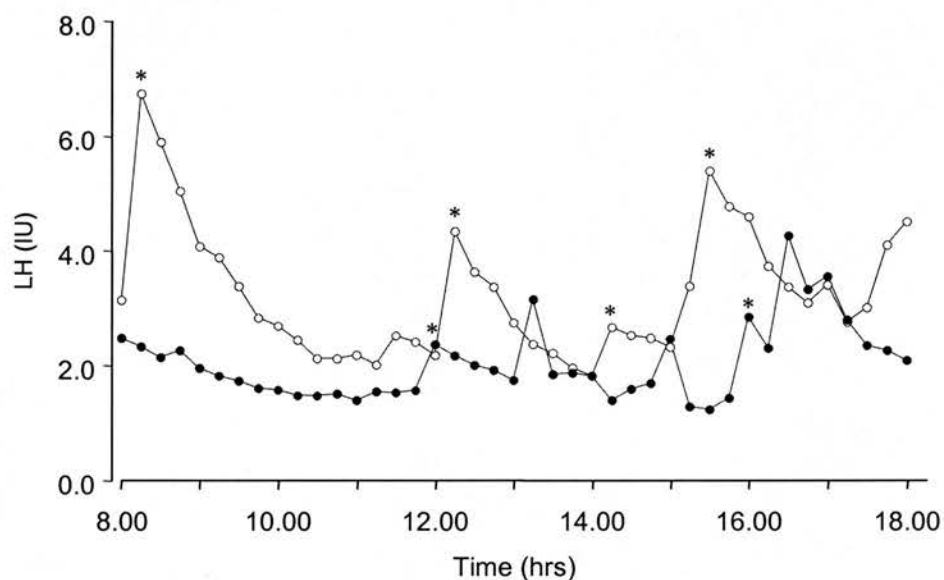


Fig 5.1 (a) Mean LH pulse amplitude (IU/L), \square Control and \blacksquare following 7 days treatment with im progesterone or oral desogestrel as indicated, * $p < 0.05$ versus control. (b) Mean LH pulse frequency (pulses/10hrs), \square Control and \blacksquare following 7 days treatment with im progesterone and oral desogestrel as indicated, $p < 0.005$ versus control.

(a) Progesterone



(b) Desogestrel

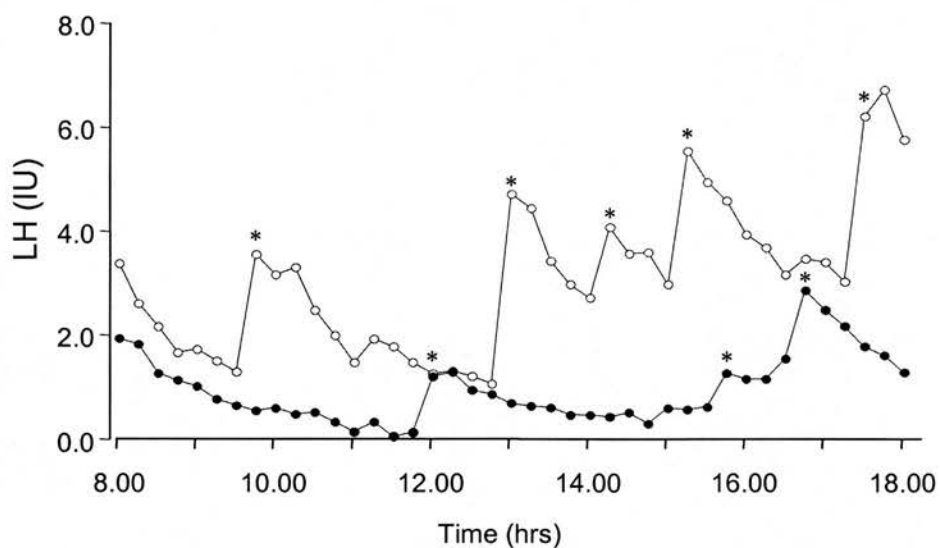


Fig 5.2. Pulsatile LH secretion pre-GnRH administration in representative individual subjects from (a) progesterone and (b) desogestrel groups. \circ Control and \bullet following 7 days treatment, * denotes pulse.

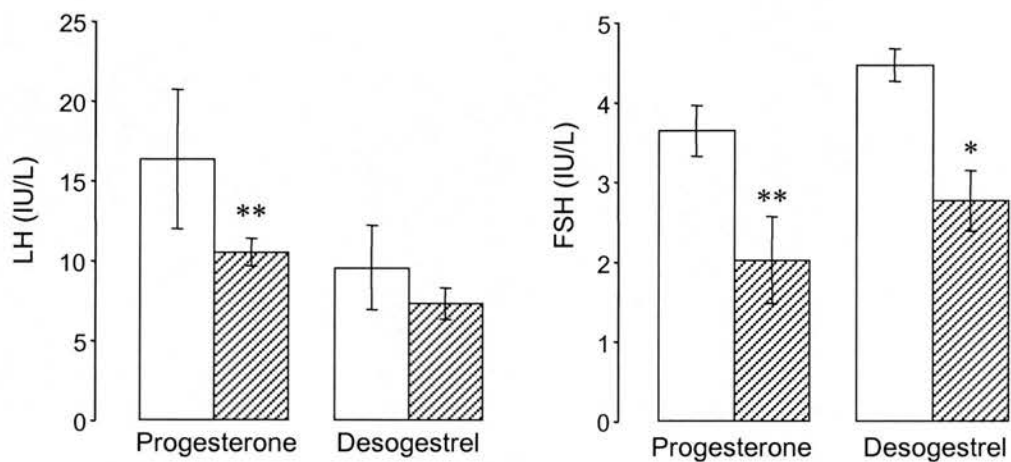


Fig 5.3. Mean (a) LH and (b) FSH response following GnRH administration after 10 hours of frequent blood sampling. □ Control and ▨ following 7 days treatment with improgesterone or oral desogestrel as indicated. * $p < 0.05$ and ** $p < 0.005$ versus control.

to 2.8 ± 0.4 ($p=0.04$), and a non-significant fall in LH from 9.5 ± 0.9 to 7.3 ± 1.0 IU/L ($p=0.13$) (fig 5.3).

5.4 Discussion

These results demonstrate the antigonadotrophic action of progesterone and desogestrel in healthy men. A dose of $300\mu\text{g}$ was chosen for desogestrel as this has been demonstrated to be optimal in male contraceptive regimes giving mean serum levels of $>1000\text{pg/ml}$ (Wu *et al.*, 1999) with no added benefit from a further increase in dose to $450\mu\text{g}$ (Bellis *et al.*, 1996). Both progesterone, the natural steroid, and synthetic desogestrel significantly decreased both LH and FSH concentrations, and the secretion of testosterone. Both steroid treatments reduced the pulse amplitude of LH secretion, and progesterone also significantly reduced LH pulse frequency. Since this model does not allow dissection of the hypothalamic-pituitary axis, these results do not allow us to determine their level of action.

In common with other steroids, gestogens bind to a variety of receptors with differing affinity. Receptor-binding studies have compared the relative binding affinities of both progesterone and desogestrel (Pollow *et al.*, 1989; Phillips *et al.*, 1990). Although desogestrel has a high selectivity index for the progesterone receptor in comparison to older synthetic gestogens, it still has significant affinity for the androgen receptor while the natural steroid demonstrates negligible binding. In view of the relative binding affinities of both treatments for the androgen and progesterone receptor, these results suggest that the antigonadotrophic effect of gestogens including desogestrel is unlikely

to be mediated solely due to their androgenicity. However, these results do not positively demonstrate that the suppression of gonadotrophin secretion by desogestrel is mediated by the progesterone receptor.

Previous studies of steroidal feedback on the hypothalamic-pituitary axis in the male have focused on the effects of exogenous androgens (Matsumoto and Bremner, 1984; Finkelstein *et al.*, 1991a; Hayes *et al.*, 2001) and more recently oestrogens (Finkelstein *et al.*, 1991a; Handelsman *et al.*, 2000; Hayes *et al.*, 2000) with relatively little data regarding the effects of progestogens. In the female, the antigonadotrophic effects of synthetic gestogens have been investigated (Couzinet *et al.*, 1996) and shown to be mediated through the progesterone receptor: the suppressive effects of synthetic gestogens on LH and FSH were not reversed on selectively blocking the androgen receptor with flutamide and their antigonadotrophic action was similar in both healthy subjects and those with complete androgen insensitivity.

Consistent with other studies in normal men (Bellis *et al.*, 1996; Wu *et al.*, 1999; Anawalt *et al.*, 2000; Kinniburgh *et al.*, 2002), desogestrel treatment resulted in a significant decline in both LH and FSH secretion. The significance of the fall in LH is evident in the concomitant decrease in testosterone concentrations. Despite this decreased endogenous gonadal steroid feedback, both treatments still resulted in suppression of LH and FSH secretion. Indeed, the degree of suppression of gonadotrophins is likely to have been yet greater if testosterone levels had been maintained by exogenous administration (Wu *et al.*, 1999). As expected from some male hormonal contraceptive studies (Martin *et al.*, 2000b; Kinniburgh *et al.*, 2002) and despite the fall in FSH secretion, serum inhibin B concentrations were unchanged after

a week of treatment in either group. Consequently, the decline in FSH cannot be attributed to any change in feedback inhibition from inhibin B.

It is difficult to ascertain at what level gonadal steroids and their synthetic derivatives exert their inhibitory effect on gonadotrophin secretion, as this is dependent on the integrated response of both the hypothalamus and pituitary. In this study, two means of indirectly assessing GnRH secretion were used. Firstly peripheral LH was used as a surrogate marker for GnRH. Portal catheterisation models in the sheep have demonstrated that the frequency of pulsatile LH secretion is closely synchronised to hypothalamic LHRH secretion (Clarke and Cummins, 1982). Interpretation of the data presented in this study is based on the assumption that all major secretory episodes of gonadotrophin were detected. Factors affecting pulse detection include assay precision, method of pulse detection and most importantly the frequency of blood sampling. A direct correlation is seen between the sampling interval used and the interpulse interval in early follicular phase in women (Crowley *et al.*, 1985): optimal is generally considered to be about 10 minutes. Although there is marked variability in normative data of LH pulsatility in normal men, sampling every 10 minutes over a 24 hour period gave a mean pulse frequency of 12.0 ± 1.1 (range 7-17 pulses/24hours) (Crowley *et al.*, 1985). Comparison to 24 hour studies is of course limited by circadian rhythm, as similar to puberty, the predominance of pulses may occur at night with clustering occurring during sleep which would not have been observed in our study. However, these published data are similar to the frequency of pulsatile LH secretion pre-treatment in the present study, and differences were detected in both progesterone and desogestrel groups (Fig 5.1b). It is possible that more frequent sampling intervals over a longer

period would have improved pulse detection in the desogestrel group; however, the low control baseline may have contributed to this result.

The second method is through the measurement of LH pulse amplitude. This however is more complex; in addition to reflecting the quantity of GnRH release it is also influenced by the effect of endogenous gonadal steroids on the sensitivity of the pituitary. A hypothalamic site of action may be suggested by the significant decline in LH pulse frequency in the progesterone-treated group (figures 5.1 & 5.2). Indeed progesterone receptors have been identified in the hypothalamus of non-primate (Lauber *et al.*, 1991; Turner *et al.*, 2001) and primate (Bethea *et al.*, 1992) animal species. Interestingly, the suppressive effect of progesterone in the ram was reduced following castration (Turner *et al.*, 2001) perhaps suggesting an interaction between progesterone receptor expression and that of other gonadal steroids, as in the endometrium. There are no data localising progesterone receptors in the human hypothalamus. The reason for the smaller, non-significant, decline in the desogestrel group is unclear but may be related to the slightly lower basal levels and frequency of LH pulses in this group. While the variance was higher in the progesterone group, the same result was determined using both parametric and non-parametric testing. It is unlikely that we would have seen a greater effect with a higher dose of desogestrel (Bellis *et al.*, 1996).

By giving a bolus of GnRH, the pituitary reserve was also examined. However, it cannot be assumed that the response to exogenous GnRH approximates that of endogenous GnRH. Conversely, any effect at the level of the pituitary cannot be presumed to result from a direct effect of the administered steroid as it may be

secondary to altered endogenous GnRH secretion. A significant attenuation of the pituitary response (both LH and FSH) to GnRH was observed in the progesterone-treated group. However in the desogestrel-treated group, a significant attenuation of only the FSH response was seen. Without a 'hypothalamic clamping' model it cannot be concluded that this is a direct pituitary effect of treatment. Progesterone receptors have been localised by immunohistochemistry to the gonadotroph of the human female pituitary gland (Couzinet *et al.*, 1999). However, a sex related difference in the distribution of the progesterone receptor is evident in the rat (Rainbow *et al.*, 1982), which may be also true of humans and there are no data at present directly demonstrating the presence of the progesterone receptor in the human male pituitary gland.

One possible mechanism whereby progesterone and synthetic gestogens may alter gonadotroph function is through decreasing the number of GnRH receptors in the pituitary. This is supported by evidence both *in vitro* and *in vivo*. In the orchidectomised sheep, progesterone stimulation reduces the concentrations of the GnRH receptor and GnRH receptor mRNA in pituitary tissue (Sakurai *et al.*, 1997). Similarly, progestin-induced falls in concentrations of GnRH receptor and receptor mRNA have been noted in ovine pituitary cells in culture (Phillips *et al.*, 1988; Laws *et al.*, 1990; Wu *et al.*, 1994). Progesterone may regulate gonadotrophin synthesis at both transcriptional and post-transcriptional levels. The 5' flanking region of the ovine gonadotropin FSH-beta gene has several progestin response elements (Webster *et al.*, 1995) and progesterone has been reported to shorten the length of the poly (A) tail of mRNA encoding gonadotrophin subunits (Wu and Miller, 1991). Therefore,

progesterone may affect the steady-state concentration of gonadotrophin mRNA by altering both the rate of gene transcription and the degree of mRNA stability. This may be the mechanism for the clear suppression of FSH that is evident with both progesterone and desogestrel. Although 3-keto-desogestrel exhibits weak glucocorticoid activity, it is unlikely that gonadotrophin receptor-mediated activity could account for the antigonadotrophic effects observed. Progesterone has low affinity and desogestrel has weak antagonistic effect at the glucocorticoid receptor (Fuhrmann *et al.*, 1995). Furthermore, it is glucocorticoid excess that has been associated with impairment of the hypothalamic-pituitary axis (MacAdams *et al.*, 1986).

In conclusion, these data demonstrate progesterone receptor-mediated suppression of gonadotrophin secretion in normal men. The demonstration that progesterone had comparable effects on gonadotrophin secretion to desogestrel indicates that synthetic gestogens exert their antigonadotrophic effect through their progestogenic properties. The relative contribution of their androgenicity was not examined. The level of interaction at the hypothalamic-pituitary axis remains unclear, although both may be involved. These data are of importance in determining the mechanism of action of agents used in male contraception. The further elucidation of their mechanism of action may enable development of regimes with increased tissue selectivity and thus greater specificity of action with fewer side effects.

CHAPTER 6

CONCLUSION

The studies presented in this thesis have demonstrated the antigonadotrophic and subsequent profound suppressive effects on spermatogenesis of the combined regimen of etonogestrel, the active metabolite of the synthetic gestogen desogestrel, in combination with different androgen preparations. The primary objective of the studies undertaken was to investigate the extent of the effects of these regimens on the suppression of gonadotrophins and spermatogenesis and secondary to further elucidate their safety and pharmacokinetics. We also aimed to compare different delivery systems both for progestogen (oral and subcutaneous implants) and androgen (intramuscular testosterone decanoate and depot testosterone pellets). The underlying mechanism of action of this regime remains unclear and the antigonadotrophic action of desogestrel was further investigated by comparing the effects of the synthetic progestogen desogestrel with progesterone on the secretion of both LH and FSH.

In chapter 2, data from the Edinburgh cohort of a multicentre study investigating oral etonogestrel with testosterone decanoate are presented. A profound and rapid suppression of spermatogenesis was evident. Group I received 300µg oral etonogestrel with 400mg TD/4 weekly with 8/10 subjects achieving azoospermia by week 24 (all < 1M/ml) increasing to 100% azoospermia by the end of the 48 week treatment period. The extent and the pattern of suppression of spermatogenesis in group II receiving the same gestogen with 400mg TD/6 weekly is clearly different, with only 4/8 subjects achieving azoospermia at week 24 (6/8 < 1M/ml) increasing to 75% azoospermia by week 48 (7/8 < 1M/ml). Gonadotrophins were also less consistently suppressed in that

group, and pharmacokinetic analysis demonstrated that although supraphysiological peaks were avoided, mean trough testosterone concentrations were subphysiological for the entire treatment period in that group, which may have contributed to this effect.

A similar pattern of suppression is observed in the data presented from the multicentre study in Chapter 3 in which 130 subjects were treated with etonogestrel implants (204mg) with the same long-acting ester, testosterone decanoate. Although direct comparisons cannot be drawn due to the largely discordant sample sizes, a greater and more rapid pattern of suppression is observed in groups I (400mg TD/4 weekly) and III (600mg TD/6 weekly) in comparison to group II that received the lower dose of testosterone decanoate (400mg TD/6 weekly). By week 24 approximately 70% of subjects were azoospermic ($90\% < 1\text{M/ml}$) in groups I and III, whereas in group II only 55% of subjects were azoospermic by that time ($80\% < 1\text{M/ml}$). During the later phase of treatment, ongoing suppression of spermatogenesis was evident with this difference in suppression between groups narrowing. Using the threshold of less than 1M/ml , 90-100% of subjects in all treatment groups achieved this by week 48. Similar to group II in the preceding study in Chapter 2, the subjects in this group demonstrated a less consistent suppression in gonadotrophins with persisting subphysiological mean trough testosterone concentrations. Clearly the androgen administered in both these groups is sub-optimal and this is reflected in the less efficacious suppression of both gonadotrophins and spermatogenesis.

Thus, a similar efficacy and pattern of suppression of spermatogenesis is observed with both oral and subcutaneous etonogestrel, in combination with testosterone decanoate. Although serum etonogestrel concentrations demonstrated wide variability in chapter 2,

previous published data report mean concentrations of 1200pg/ml with 300µg po desogestrel (Wu *et al.*, 1999a) and 500-800pg/ml with 150µg po desogestrel (Anawalt *et al.*, 2000). Therefore, dose sparing is evident with the implant preparation, with mean concentrations of 300-800pg/ml achieving equivalent biological effect with respect to suppression of spermatogenesis.

Without a placebo group and with largely discordant sample sizes, it is not possible to compare or conclude on whether this 'dose sparing' is reflected in the non-reproductive and side-effect profiles of these two studies. Consistent with data from previous studies investigating desogestrel, a decline in HDL-C (Wu *et al.*, 1999; Anawalt *et al.*, 2000) and increase in weight (Anawalt *et al.*, 2000) was evident with both the studies with oral and subcutaneous preparations. Few studies to date have investigated the effects of progestogen/androgen regimes on body composition. Interestingly the significant increase in weight in group I in chapter two seemed to reflect an increase in fat free mass, in agreement with observations with androgen and levonogestrel combinations (Herbst *et al.*, 2003). Significant increases in haemoglobin in groups I of both studies and also haematocrit in group I of the oral etonogestrel study were observed, which may reflect high testosterone peaks with the higher dose of this ester.

An alternative approach was considered in chapter 4. We demonstrated that three etonogestrel implants (204mg) in combination with 400mg depot testosterone pellets administered 12 weekly improved upon previous efficacy in suppression of spermatogenesis with 1 or 2 implants and the same androgen preparation (Anderson *et al.*, 2002a). Rapid and profound suppression of spermatogenesis was observed with azoospermia in all 14 subjects compared to 64% and 75% in our previous study with

one and two implants respectively. Although a similar proportion of subjects were azoospermic at week 24 (11/14, 78%) to the previous studies, all subjects at week 16 had concentrations of less than 1M/ml, exhibiting a more rapid suppression of spermatogenesis. Eight of the nine subjects who continued the study maintained azoospermia for 48 weeks of treatment. This increased efficacy is also supported by the finding of consistent suppression of gonadotrophins and both serum and seminal plasma Inhibin B. Dose sparing is also evident with suppression of spermatogenesis to similar efficacy as observed with 300µg desogestrel per day with lower overall mean etonogestrel concentrations of 600pg/ml. This is equivalent to levels observed with the suboptimal oral dose of 150µg desogestrel per day. In contrast to the preceding studies, testosterone concentrations remained within the physiological range throughout treatment. Testosterone pellets, although requiring surgical insertion, may confer significant advantages over long-acting esters and demonstrate smoother pharmacokinetic profiles with near zero-order release (Handelsman *et al.*, 1990) allowing further dose-sparing. This may have contributed to the minimal adverse metabolic effects observed in this study in comparison to the studies in chapters 2 and 3. No significant weight gain, body composition or decline in HDL-C concentrations were found.

As was the case with the female combined oral contraceptive pill, at the time of marketing the long term effects of male contraceptive regimes will be unknown, especially regarding cardiac and prostate disease. Indeed, using a testosterone dose in the low physiological range as with the approach in Chapter 4 may be of importance in the avoidance of potential harm with a future product. The dose of androgen replacement is usually considered to equate to peak concentrations observed in the

morning, resulting in an overestimation of requirements for physiological replacement. The data presented in Chapter 4 confirms that the diurnal variation of testosterone is lost during administration of exogenous androgen/progestogen regimens. We proposed that the dose of pellets administered in this study more closely replace testosterone production based on physiological diurnal variation rather than morning peaks and this may have contributed to the lack of change in non-reproductive functions such as haematocrit that is observed, and the enhanced suppression of spermatogenesis (Meriggiola *et al.*, 2002; Zhang *et al.*, 2003).

Having demonstrated the suppressive actions of gestogen/androgen regimes on the secretion of gonadotrophins and spermatogenesis, the underlying mechanisms of action were further investigated. Synthetic gestogens have affinity for both androgen and progesterone receptor and the relative contribution of action of these receptors in the suppression of gonadotrophins in the male are unclear. In chapter 5 the effects on gonadotrophin secretion of desogestrel, which has affinity for both the androgen and progesterone receptor were compared with the naturally occurring steroid progesterone, which has relatively no affinity for the androgen receptor. Both steroids suppressed the secretion of LH, FSH and testosterone. Both reduced LH pulse amplitude, and progesterone reduced LH pulse frequency. Progesterone reduced the increase in LH secretion in response to a GnRH bolus. We demonstrated that the suppressive effects of desogestrel on gonadotrophin secretion are as significant as those of progesterone and conclude that they exert this effect through their progestogenic properties. We were unable to determine from this model the relative contribution of their androgenicity or their level of action at the hypothalamic-pituitary axis. It would be helpful in developing male contraceptives to further elucidate their underlying mechanisms of

action at the hypothalamic-pituitary and testicular level. This will enable the development of regimes with increased tissue selectivity, increased specificity of action and thus fewer side effects.

With advances in cellular and molecular biology the development of selective modulators of both androgen and progesterone receptors may help achieve this goal leading to a method which specifically suppresses spermatogenesis whilst maintaining muscle and bone mass and having no adverse effects on the prostate or lipid metabolism. The existence of multiple receptors as demonstrated for oestrogen, ER α and ER β (Kuiper *et al.*, 1996), may also be true for androgens but have not been identified. A group of molecules have been identified which have selectivity and specificity for the AR (Edwards *et al.*, 1999) and their further modification may lead to them displaying agonist, antagonist or partial effects (Negro-Vilar, 1999). Recently, a non-steroidal selective androgen receptor modulator has been shown to significantly lower gonadotropins and suppress spermatogenesis in male rats (Chen *et al.*, 2004). This may not only be of great benefit in the area of male hormonal contraception but also may have more widespread clinical applications in the treatment of hypogonadism, androgen-dependent malignancy and in the development of HRT in ageing men. Similarly the development of specific orally active progesterone receptor agonists may replace conventional progestogens as an adjuvant in male contraceptive strategies (Edwards *et al.*, 1998; Zhi *et al.*, 1998).

Of the different approaches to male contraception, the closest to practical implementation is the hormonal approach. However, there is clearly a need for further longer term large-scale studies to further evaluate the efficacy and safety of these

regimes. Further understanding of the process of spermatogenesis from the level of germ cell proliferation through epididymal maturation will diversify the potential targets for contraception in the male.

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Expert Opinion

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Monthly Focus: Endocrine, Metabolic & Drug Delivery

Advances in male contraception

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Advances in contraception have been almost exclusively female-directed despite the widespread use of male methods worldwide and increasing calls for the burden of contraception to be more evenly shared. Of the several potential approaches to novel male methods, the hormonal approach is the nearest to fruition. The use of testosterone as a reversible contraceptive agent in men has been demonstrated in studies undertaken by the WHO over the last decade. However, an agent that results in universal azoospermia without significant side effects remains elusive. Consequently, combination approaches with progestogens, anti-androgens, 5 α -reductase inhibitors and gonadotrophin releasing hormone (GnRH) antagonists have been evaluated with the aim of improving contraceptive efficacy. Different methods of androgen delivery are also being developed in order to minimise extra-testicular effects and improve acceptability. This review will focus on efforts to develop a safe, acceptable, efficacious hormonal contraceptive for men.

Keywords: contraceptive efficacy, male hormonal contraception, progesterone analogues and derivatives, spermatogenesis, testosterone analogues and derivatives, testosterone metabolism, actions and pharmacology

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1. Introduction

With one third of the world's population relying on male methods of contraception, namely condoms, coitus interruptus and vasectomy [1], there is a need to expand the choices available for these couples. The concept of male hormonal contraception is by no means new. As early as the 1930s, reversible inhibition of spermatogenesis with little effect on libido was demonstrated [2] with the administration of testosterone. Since then, many attempts have been made at providing a safe, reversible means of inducing azoospermia, without any long-term adverse effects on health. In addition to issues of safety and efficacy, acceptability and speed of onset of action must be considered. However, progress over the decades has been slow and pharmaceutical industry interest and funding has been limited. Furthermore, our incomplete knowledge of male reproductive physiology has limited the potential targets for inhibition of fertility. These targets can be classified as follows:

- pre-testicular: withdrawal of gonadotrophin support of the testis (luteinising hormone [LH] and follicle stimulating hormone [FSH])
- testicular: interfering with spermatogenesis at the level of the seminiferous tubules
- post-testicular: disrupting sperm maturation and transport, particularly at the level of the epididymis

The testicular and post-testicular approaches have several theoretical advantages, including specificity of action to the reproductive system, testosterone replacement not being required, and rapidity of onset. Difficulties remain in identifying aspects of testicular or epididymal function without toxicity. The most widely tested compound in this respect has been gossypol, derived from cotton seed. While this agent resulted in a very high prevalence of azoospermia, this was in many cases irreversible

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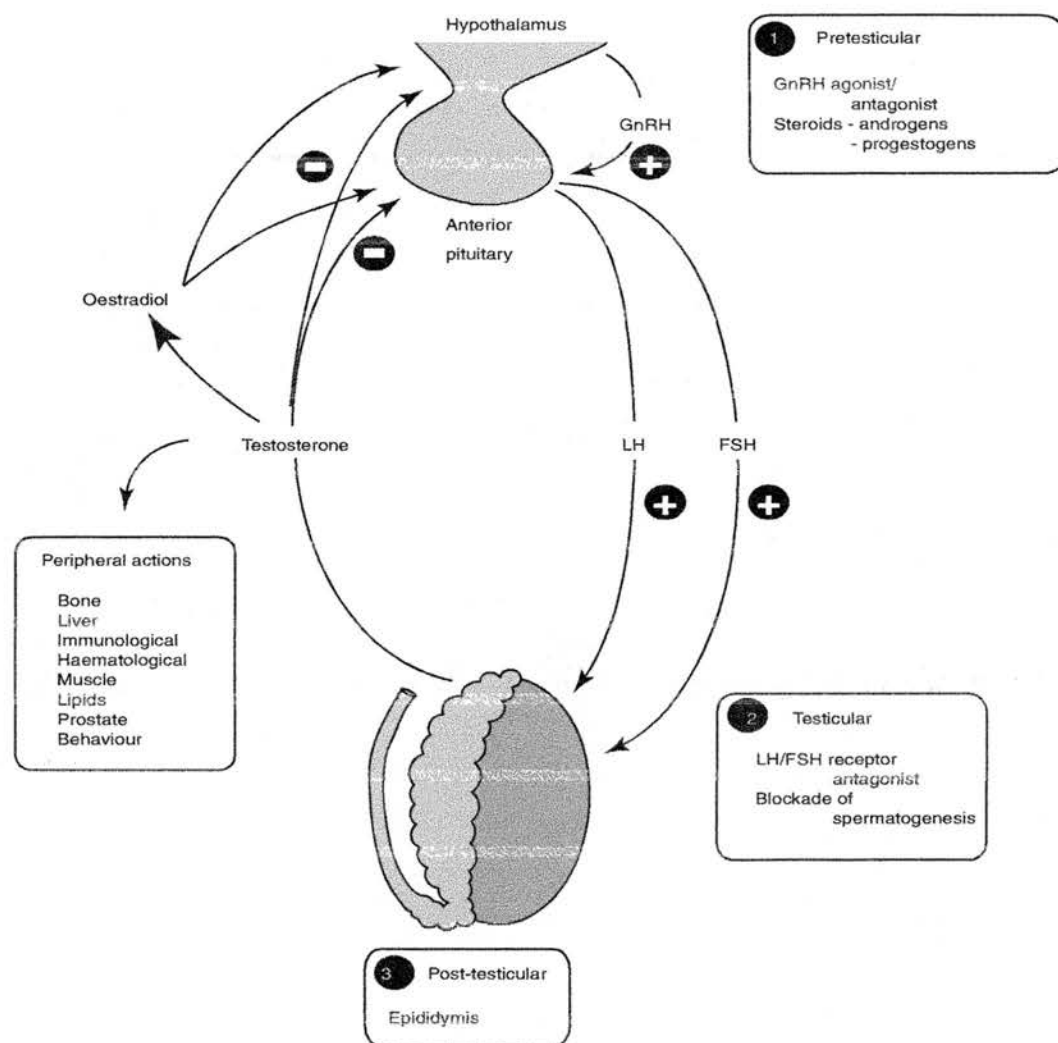


Figure 1. The hypothalamic-pituitary-gonadal axis in men. Potential targets of male contraception. FSH: Follicle stimulating hormone; GnRH: Gonadotrophin releasing hormone; LH: Luteinising hormone.

and there were other side effects, including hypokalaemia [3]. It is, however, likely that with increasing understanding of the molecular basis of spermatogenesis and sperm function, that an appropriate target will be found. Recently identified molecules which may be potential targets include ion channels specifically expressed by sperm [4], and molecules involved in the interaction between germ cells and Sertoli cells [5]. The focus of this review will be on the pre-testicular, hormonal approach to male contraception, as it is the one area which has progressed to clinical studies.

2. Hormonal approaches

The revolution in female contraceptive provision, enabled by advances in steroid chemistry in the second half of the last century, have focused attention on the analogous targets in the male, i.e. the administration of exogenous hormone to inhibit the production of gonadotrophins at the level of the hypothalamus and pituitary gland. Spermatogenesis is dependent on a sufficient concentration of testosterone within the testis, the physiological concentrations of which are 50- to 100-fold higher than in peripheral blood [6,7]. Ster-

oidogenesis is stimulated by the action of LH on the Leydig cells of the testis, which acts in concert with FSH via the Sertoli cells to ensure quantitatively and qualitatively normal spermatogenesis. Inhibition of both FSH and LH is required for adequate inhibition of spermatogenesis, but the fall in testicular steroidogenesis will result in symptoms of hypogonadism and in the longer term decreased bone mass, muscle bulk and deficient haematopoiesis. Therefore, androgen replacement remains an essential component of all male hormonal contraceptive regimes. While supraphysiological doses of testosterone will suppress spermatogenesis by inhibiting gonadotrophin secretion, an approach demonstrated to be an effective contraceptive (as discussed below), it may also paradoxically support spermatogenesis, although this has been much more clearly demonstrated in rodents than in man. Therefore, the aim is to provide sufficient testosterone to suppress gonadotrophin production and prevent hypogonadism without stimulating spermatogenesis. The co-administration of a second agent, such as a progestogen or GnRH analogue, makes this much more readily achievable. While testosterone administration alone is not a feasible contraceptive, much valuable data has come from studies using this approach.

2.1 Testosterone enanthate: proof of concept

The degree of reduction in sperm concentration from the normal of 20 - 200 million/ml required to achieve acceptable contraceptive efficacy is uncertain. Indeed, pregnancies have been seen in partners of men taking part in contraceptive studies whose sperm concentration was suppressed to below 1×10^6 /ml, although exposure was for many months prior to conception [8]. This was specifically investigated in two studies carried out by the WHO designed to investigate the degree of spermatogenic suppression required for contraceptive efficacy. These studies used a prototype regimen of testosterone enanthate (TE) administered weekly and were based on encouraging results from the 1970s [9,10], when over half Caucasian subjects attained azoospermia, the remainder becoming oligozoospermic.

The first WHO study investigated efficacy in subjects once they had achieved azoospermia. A subsequent larger study included all men whose sperm concentrations had fallen below 3×10^6 /ml. Both studies were based on weekly administration of 200 mg TE im. There were no pregnancies per 230 person-years in the azoospermic group. There were, however, a small but significant number of pregnancies in the oligozoospermic group, giving an overall pregnancy rate of 1.4 per 100 person-years (CI 0.4 - 3.7) [11,12]. The pregnancy rate in those men with sperm concentrations between $0.1 - 3 \times 10^6$ /ml was 8.1 per 100 person-years (95% CI 2.2 - 20.7). Of the subjects, 98% actually entered the 12-month efficacy phase of the study, achieving concentrations of $< 3 \times 10^6$ within 6 months of treatment. Therefore, the overall failure rate, including those who did not achieve oligozoospermia, was 3.4%, giving an overall contraceptive efficacy of 96.6%. These studies served as a clear demonstration that steroid-

induced suppression of spermatogenesis is a possibility for a contraceptive method as it is effective and entirely reversible. However, the significant risk of pregnancy in the oligozoospermic group emphasises the need to develop a method achieving more uniform azoospermia. Supraphysiological testosterone levels also resulted in potentially adverse metabolic effects, particularly a decrease in high-density lipoprotein cholesterol (HDL-C), a risk factor for ischaemic heart disease [13-15], although this was not seen in the Chinese subjects [16]. A striking feature from these studies is the ethnic heterogeneity in the degree of suppression of spermatogenesis, with Asian men consistently showing a higher prevalence of azoospermia than Caucasian men [17]. The underlying mechanisms have been extensively investigated and at present remain unclear [18,19]. These important studies therefore confirm the concept of hormonal suppression for contraceptive efficacy. While available testosterone preparations remain unsatisfactory, the development of long-acting testosterone preparations is required to exploit this demonstration of proof of concept.

2.2 Androgen/progestogen combinations

Progestogens are potent inhibitors of gonadotrophins in both men and women. Thus, in combination with testosterone, they allow lower doses of each steroid to be given. Consequently, androgen-associated side effects and ongoing androgen-supported spermatogenesis are avoided. A major advantage is their availability, as they have been used for several decades as components of female hormonal contraception, and the range of existing products can be administered orally, im. or by sc. implants.

Depot medroxyprogesterone acetate (DMPA) has been widely investigated as the progestogen component. In a series of studies in the 1970s, > 100 men were given monthly DMPA injections in combination with monthly TE 100 - 250 mg for up to 16 months [20-24]. Although azoospermia was achieved in half the subjects, contraceptive efficacy was poor with several resulting pregnancies. Side effects from this regime included weight gain, gynaecomastia and a transient decline in HDL-C. Similar to androgen-only regimes, ethnic polymorphism in spermatogenic response is evident with androgen-progestogen combinations. A higher prevalence of azoospermia was found in studies in Indonesian men, with 97% achieving azoospermia using DMPA with 19-nortestosterone or TE [25]. The high prevalence of azoospermia in Indonesian men with testosterone-only regimens, however, means that the contribution of the DMPA in that study is unclear. Doubling the dose of TE did not increase efficacy of this regime in Caucasian men [26]. However, administration of testosterone pellets (800 mg) with 300 mg DMPA (single administration only) resulted in a high incidence of azoospermia, with 9/10 men becoming azoospermic compared to 4/10 with testosterone alone [27]. This regimen, i.e. depot preparations of both testosterone and DMPA, is currently under further investigation in Australia (DJ Handelsman, personal communication).

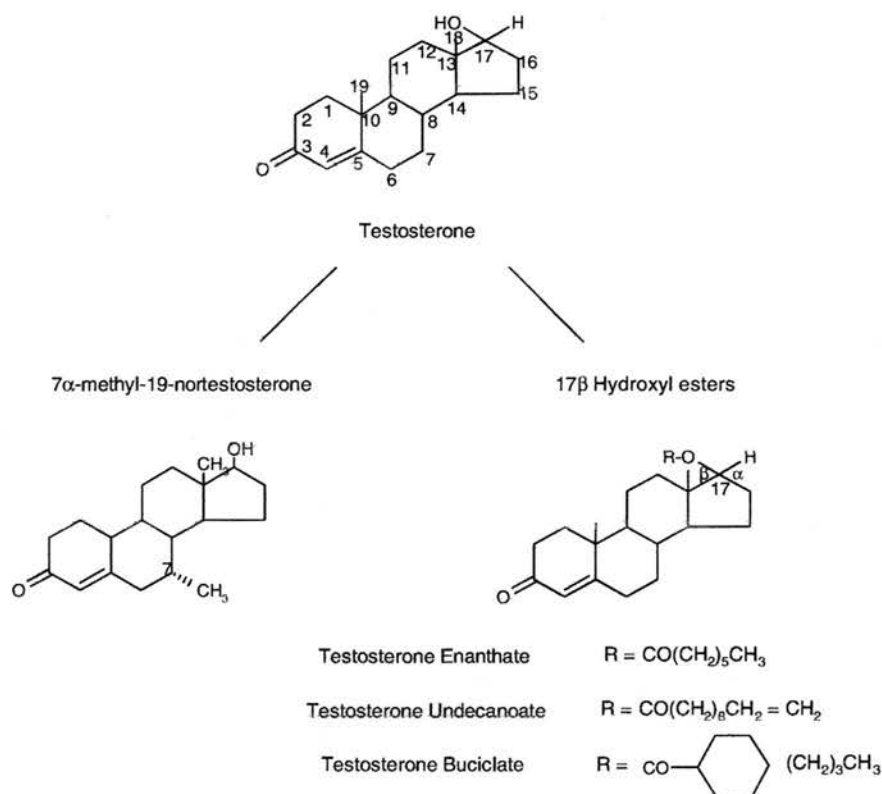


Figure 2. Molecular structures of testosterone and derivatives currently used in male contraceptive studies.

Levonorgestrel has also been widely investigated in combination with testosterone. Results from earlier studies, using a combination of oral levonorgestrel with TE, were encouraging. Administration of 250 - 500 µg levonorgestrel with low dosage TE (200 mg/month) suppressed sperm concentration in half the subjects below $5 \times 10^6/\text{ml}$, but no subjects reached azoospermia [28]. Recent randomised controlled trials with more frequent testosterone dosing not only showed increased suppression of spermatogenesis but also demonstrated that the levonorgestrel-TE combination was superior to using androgen alone [29]. Administration of 500 µg levonorgestrel with 100 mg TE weekly resulted in 94% of subjects becoming azoospermic or severely oligozoospermic in comparison to 61% in the TE group alone. In addition, the time to onset of azoospermia was more rapid in the combined group. However, similar to previous progestogen combinations there was a significant reduction in HDL-C of approximately 20% and subjects also experienced weight gain. Subsequent studies lowering the dose of levonorgestrel to 125 µg orally, resulted in a slight reduction in suppression of spermatogenesis (78% compared to 89% with 500 µg), but reduced weight gain and HDL-C suppression were observed [30].

Another approach has recently been reported combining a long-acting depot injection of testosterone undecanoate

(TU) with the progestogen norethisterone (NET) enanthate. NET enanthate has been established as a depot contraceptive in women [31], currently licensed in the UK for short-term use. NET enanthate has been shown to have a profoundly suppressive effect on gonadotrophins in men [32] and demonstrates some unique biochemical properties. NET binds to the androgen receptor with 45% the affinity of testosterone, resulting in androgenic activity of about 10% that of testosterone [33], which may be of further benefit in male contraception. It undergoes 5α-reduction to 5α-NET, enhancing its relative binding affinity for the androgen receptor but unlike DHT, paradoxically diminishing its androgenic potency in target organs [34], such as the prostate. Used as a contraceptive agent in men, the injection intervals were conveniently equal for both steroids (6 weekly) and results from the combination were superior to TU alone with 13 out of 14 compared to 7 out of 14 men becoming azoospermic [32].

Perhaps the most promising results to date using this combination have been obtained with the synthetic progestogen desogestrel [35,36]. In one study, eight out of eight men became azoospermic with oral desogestrel 300 µg daily in combination with a weekly dosage of 50 mg TE im. [35]. This study also demonstrated the narrow dose-response relationship with

this combination: either decreasing the dose of desogestrel to 150 µg or a higher dose of testosterone (100 mg/week) resulted in a lower apparent incidence of azoospermia although the groups were of small size. In a further study, 94% of men became azoospermic on receiving 150 or 300 µg desogestrel in combination with 100 mg TE weekly. Once again, the narrow dose-response was evident, with reduction of the dose of testosterone reducing levels of suppression. Desogestrel also had a dose-dependent effect on lowering of HDL-C and weight gain [36]. In a dose-finding study, 300 µg desogestrel with testosterone pellets resulted in greater spermatogenic suppression than 75 or 150 µg [37]. We have recently demonstrated that this combination resulted in azoospermia in all subjects investigated in both Scotland and Shanghai [38]. As with other testosterone/gestogen preparations, weight gain and HDL-C suppression were reported and were demonstrated to be dependent upon the doses of both desogestrel and testosterone.

Long-acting progestogen implant preparations offer another attractive method of delivery. In addition to not being user-dependent, a more sustained release preparation may allow dosage reduction and avoid fluctuating serum steroid levels that may result from oral treatments. However, little current data is available. Administration of levonorgestrel implants in combination with injectable testosterone undecanoate (250 mg/month) resulted in 6 out of 18 Chinese subjects becoming azoospermic [39]. However, results have been more encouraging with Implanon® (Organon, NV), which releases etonogestrel, the active metabolite of desogestrel. Results from administration of 1 implant, similar to that used in women, in combination with testosterone pellets were disappointing. However, increasing the dose to 2 implants greatly increased suppression of spermatogenesis, with 13/14 men achieving sperm concentrations $< 0.1 \times 10^6/\text{ml}$ [40].

To date several androgen/gestogen combinations have been used with a significant improvement in efficacy from previous androgen-only approaches. However, extra-testicular side effects, namely on lipid metabolism and with weight gain remain a problem which future regimes will aim to minimise.

2.3 Androgen/anti-androgen combinations

Administration of an anti-androgen might appear inappropriate in a hormonal regimen that requires maintenance of peripheral androgen action. However, inhibition of the action of residual intratesticular concentrations of testosterone may increase efficacy in attaining azoospermia. Cyproterone acetate (CPA), an anti-androgen and progestogen, has been investigated as a potential male contraceptive. Administration of CPA alone at a dose of up to 10 mg/day resulted in a moderate reduction in sperm production, but a marked suppression in androgen levels and libido [41]. Subsequent studies in India of CPA in combination with TE, rather than CPA alone, resulted in azoospermia in the majority of subjects with no major adverse effects [42]. This combination has been recently reinvestigated, 5 patients in each group receiving 50

or 100 mg CPA daily orally with 100 mg TE weekly, or TE alone at the same dose [43]. The adjuvant benefit of CPA was apparent with all subjects in those groups attaining azoospermia, whereas only 3/5 of the testosterone alone group exhibited similar suppression. Subsequent dose-finding studies demonstrated high efficacy of CPA at doses > 25 mg/day with no adverse effect on blood lipid profile [44]. However, there was a dose-dependent suppression of haemoglobin and haematocrit reflecting the anti-androgenic effect. With the aim of developing a 'male pill', further studies investigated a further decrease in CPA dose to 12.5 mg combined with oral testosterone undecanoate 80 mg twice daily [45]. Results were however disappointing, with azoospermia being achieved in only 2 out of 8 subjects. Again, this emphasises the narrow therapeutic index of male contraceptive regimes and the fine balance between the anti-androgenic properties of CPA and the androgenic effects of testosterone.

2.4 The potential of 5 α -reductase inhibition

The enzyme 5 α -reductase converts testosterone to DHT, which by having greater potency acts as an amplifier of testosterone action in tissues in which it is highly expressed, such as the prostate. Two isoforms of the enzyme have been identified [46]. Under physiological conditions, intratesticular testosterone concentrations are high and are presumed to saturate the androgen receptor. However, when endogenous production is low, conversion to DHT may become an important factor in the maintenance of spermatogenesis. 5 α -Reductase activity has been demonstrated in the human testis [47,48]. However, evidence is scant regarding the nature, regulation and isoform of testicular 5 α -reductase in the adult male. mRNA levels and enzymatic activity are very low in the human testis, although the presence of the type 2 enzyme isoform is suggested [49]. In contrast, type 1 is the predominant isoform in the rat testis [50]. It has been suggested that differences in 5 α -reductase activity may underlie the heterogeneity of response to TE, explaining why some individuals maintain oligozoospermia [19]. Parallels have also been drawn between 5 α -reductase activity in different ethnic groups [51,52], which may relate to the differing response between Caucasian and non-Caucasian populations discussed above. The importance of 5 α -reductase has been demonstrated in adult rats, with the inhibition of 5 α -reductase leading to an increased requirement of testosterone to support spermatogenesis. This implies that conversion of T to DHT may permit ongoing spermatogenesis when intratesticular T levels are reduced [50]. However, the extent to which 5 α -reductase supports spermatogenesis in humans is less clear. In a recent clinical study, administration of finasteride, a 5 α -reductase type 2 inhibitor, did not enhance the rate or degree of suppression of spermatogenesis in males upon administering a progestin/testosterone combined contraceptive regime [53]. Similarly, in men failing to suppress within 3 months of treatment with testosterone implants, there was no enhancement of suppression of spermatogenesis when finasteride was added [54].

Although the effect of co-administration with an inhibitor of the type 1 isoform has not been investigated, no additional benefit is evident from 5 α -reductase inhibition in a contraceptive regimen at present.

2.5 Androgen/oestrogen combinations

Evidence is now emerging to support an important role for oestrogen in spermatogenesis. In the oestrogen receptor α (ER α) knockout mouse, the seminiferous tubules are dysfunctional and the epididymis is unable to support sperm maturation with consequent infertility [55-57]. Individuals with oestrogen receptor mutations and aromatase-deficiency illustrate the importance of aromatisation in the physiology of the male [58,59]. A male reported to have a mutation in CYP19 encoding aromatase was infertile [60]. However, his brother with a normal CYP19 gene was also infertile and thus familial occurrence limits the interpretation of the impact of this mutation on spermatogenesis. Oestrogen blockade may therefore become a potential target for contraception. However, this would have to be in a selective manner in order to avoid associated adverse effects, such as osteoporosis and hyperlipidaemia.

An alternative basis for the use of oestrogen in male contraception is the demonstration that aromatisation is crucial for mediating the feedback effect of testosterone on FSH secretion [61]. The enhanced suppression of spermatogenesis resulting from the addition of low dose oestradiol to testosterone in non-human primates was demonstrated in a series of studies in the 1970s [62] and has recently been confirmed in humans [63]. However, the therapeutic margin before the occurrence of oestrogenic side effects was narrow and the levels of suppression achieved suboptimal for an acceptable contraceptive method. The administration of oestrogen also raises safety concerns with the hypothetical risk of arterial thromboembolism. It seems unlikely at present that this combination will be used, however, these data emphasise the importance of amortisation of any synthetic androgen used in this context.

2.6 GnRH agonists and antagonists

GnRH agonist analogues have become established therapies in a wide range of hormone-dependent diseases. In combination with an androgen, suppression of gonadotrophin secretion and consequent suppression of spermatogenesis is a further potential application. As the action is more specific than that observed with other steroids such as progestogens, it may diminish adverse systemic effects and lower the total dose of steroid required. However, in general, results from clinical studies involving administration of GnRH agonists *sc.* (D-Trp⁶, buserelin and nafarelin) with varying doses of TE, have been disappointing with failure of complete suppression of spermatogenesis [64]. Similar results were obtained when given as a continuous infusion. Only six out of eight subjects became oligozoospermic, with 2 retaining normal sperm densities on infusion of 500 μ g LHRH agonist daily with 100 mg TE every 2 weeks [65]. It appears that failure of spermatogenic suppression resulted from initial stimulation of gonado-

trophin secretion ('flare') and subsequent 'escape', particularly of FSH [66].

GnRH antagonists may increase the degree of gonadotrophic suppression and thus of spermatogenesis. Results from clinical studies have been more encouraging than with the agonists, with the administration of the prototype antagonist Nal-Glu with TE resulting in profound and rapid suppression of spermatogenesis [67-69]. However, progress has been limited by their histamine-like side effects upon injection, their short duration of action, and the expense involved in their synthesis. This has, however, had the effect of stimulating research into biphasic administration protocols, with one drug regimen for the suppression phase followed by a lower dose maintenance phase. This was investigated using the GnRH antagonist, cetrorelix, in combination with 19-nortestosterone (200 mg/3 weekly) [70]. All men became azoospermic with the combined drug regimen, but when cetrorelix was discontinued and the androgen continued alone, spermatogenesis was restored. This may have resulted from inadequate androgen dosage or too prolonged a dosage interval. A second study involved administration of Nal-Glu with TE for 16 weeks and induced azoospermia in 10/15 men: subsequent TE-only 'maintenance' for 20 weeks sustained suppression in 13 out of 14 subjects, with only one showing escape [71]. This dose of 100 mg TE week alone is relatively ineffective in inducing azoospermia [29]. Limiting GnRH treatment to the 'induction phase' may reduce costs as well as drug exposure. Orally active non-peptide GnRH antagonists have been described [72], but no data relevant to the present discussion are available.

3. Androgen delivery

3.1 Testosterone esters

Improving methods of androgen delivery remains one of the major hurdles in the development of male hormonal contraception. Testosterone is promptly degraded by the liver in first-pass metabolism and thus cannot be administered orally, with the exception of testosterone undecanoate. This ester is partially absorbed from the intestine in chylomicra therefore avoiding first-pass metabolism. However, it has a short duration of action with widely fluctuating plasma concentrations [73]. In addition to unpredictable pharmacokinetics with high inter- and intra-individual variation, high oral doses are required. Oral TU has been investigated with oral CPA as a 'male pill' [74], but these characteristics make it unsuitable for widespread use as a male contraceptive. The most widely used preparations, both in the above contraceptive studies and in hypogonadal replacement therapy, involve esterification of 17 β -hydroxyl group with carboxylic acids. This increases the polarity of the molecule, making it more lipophilic and hydrophobic, thereby slowing release from the injection site. Longer-acting testosterone preparations would improve the adverse pharmacokinetics of TE and similar preparations [75,76] and necessitate a lower overall dosage for equivalent efficacy, as demonstrated with testosterone pellets [77]. Testoster-

one buccinate exemplifies this approach, with a terminal half-life of 29.5 [75] *versus* 4.5 days for TE [76]. In clinical studies, a single dose of 1200 mg im. resulted in azoospermia in 3 out of 8 men while maintaining plasma testosterone levels in normal range [78]. However, it is not currently available for further investigation. The undecanoate ester can also be administered im. with improved pharmacokinetics compared to TE, providing testosterone replacement for 6–8 weeks [79–81]. In a recent clinical study in China, 11 out of 12 volunteers became azoospermic with a dose of 500 mg TU/4 weeks, whereas 12 out of 12 became azoospermic with the higher dose group (1000 mg/4 weeks) [82]. Investigation of a similar TU preparation in a Caucasian population demonstrated a lower incidence of azoospermia when given alone at 1000 mg/6 weeks [32]. However, in combination with the long-acting injectable progestin, norethisterone enanthate, 13 out of 14 men became azoospermic.

3.2 Testosterone pellets

Sc. implantation was the earliest effective modality for the clinical application of testosterone. The first pellets were made from high pressure compression and contained cholesterol. Currently available implants of fused crystalline testosterone are normally inserted surgically into the anterior abdominal wall under local anaesthesia. They have been in use since the 1950s for the treatment of hypogonadal men and are fully biodegradable. They have near complete bioavailability with kinetics approximating zero-order release [83], resulting in relatively stable serum concentrations. A dose of 800 mg (4 x 200 mg pellets) provides physiological testosterone replacement for 4–6 months releasing approximately 6 mg of testosterone per day [84] with good acceptability in hypogonadal men. In contraceptive studies, testosterone pellets alone at a dose of 1200 mg achieved an equivalent degree of spermatogenic suppression to 200 mg TE weekly, with fewer androgen-related side effects [77]. Further dose-sparing resulted in decreased efficacy with only 4 out of 10 subjects becoming azoospermic with 800 mg testosterone [27]. However, in combination with a single dose DMPA 300 mg im., the extent of suppression was markedly increased with 9 out of 10 subjects becoming azoospermic. In a recent study in our centre, the combination of 400 mg testosterone pellets every 12 weeks with oral desogestrel resulted in azoospermia in all men while maintaining plasma testosterone concentrations within the physiological range throughout [85]. However, the pellets do require a minor surgical procedure for insertion and with an extrusion rate of 7%, a longer-acting depot injection seems the optimal choice at present. However, their long duration of action and avoidance of initial supraphysiological concentrations mean that the pellets remain a valuable prototype at present.

3.3 Topical testosterone

A more recent development is the transdermal delivery of testosterone, which can be administered by scrotal and non-scro-

tal routes. In comparison to female HRT when doses in the region of 50–100 µg/day of oestrogen are used much higher amounts of testosterone require to be delivered (3–10 mg/day). Testoderm® (testosterone transdermal system, ALZA Corporation) patches in doses of 2.4 or 3.6 mg, maintain testosterone concentrations in the adult physiological range for 24 h when applied to the scrotum [86]. They also elevate the DHT/T ratio to 0.5 (normal 0.1–0.2), reflecting the rich 5α-reductase activity in the scrotum. It is possible that this would have suboptimal effects in androgen target organs, such as bone, which are predominantly dependent on aromatisation of testosterone. Similarly, non-scrotal systems have been developed for use in hypogonadal men [87]. Androderm® transdermal delivery systems (Teratech) at a dose of 2.5 or 5 mg maintain testosterone concentrations without altering the DHT/T ratio. However, in one study 72% of hypogonadal subjects elected return to depot injections as a result of dermatological problems [88]. In a recent randomised parallel group study comparing transdermal testosterone 5 mg/day with TE 200 mg im. every 2 weeks in hypogonadal men, more physiological hormone levels were reported with reduced stimulation of erythropoiesis and gynaecomastia with the topical preparation. However, once again minor skin irritation, which can be serious [90], was reported in 60% of patients [89]. A further topical method of delivery involves the application of testosterone and DHT gels. In studies using hydraalcoholic DHT gels providing metered doses of 16 mg/2.3 g gel no skin irritation was reported [91]. Applying metered doses of 16, 32 or 64 mg provides testosterone replacement in the low, middle and high physiological range. As DHT is a more potent androgen than testosterone, less drug is required. The effect on the prostate is therefore reduced, as amplification of the effect of testosterone (by conversion to DHT) is avoided [92,93]. Testosterone gels have also been shown to decrease bone resorption in hypogonadal men resulting in a significant increase in bone mineral density [94].

In the 1980s, the concept of using a self-administered contraceptive regime was investigated with daily oral MPA 20 mg with percutaneous testosterone 50/100 mg per day and resulted in a significant reduction in sperm density with no reduction in libido [95]. More recently, combination of oral desogestrel with transdermal testosterone (Andropatch®) resulted in less consistent suppression of spermatogenesis (66% with 300 µg desogestrel) than with injectable esters. Marked skin irritation resulted in a significant number of men withdrawing from the study [96]. Similarly, only 2/11 volunteers reported no skin reaction using daily transdermal patches with oral levonorgestrel (125 or 250 µg), and only 2/11 becoming azoospermic [97]. In avoiding the necessity for repetitive injections and allowing self-administration, these methods of androgen replacement may prove very acceptable for use as part of a male hormonal contraceptive. However, further work is necessary in order to avoid the high incidence of local side effects from patches and improve the degree of spermatogenic suppression.

3.4 7 α -Methyl-19-nortestosterone.

An alternate approach is the use of an androgen other than testosterone itself. Potential advantages may include increased potency and degrees of tissue selectivity, allowing smaller quantities of drug to be administered with equipotent biological effects on some tissues, such as the hypothalamus and pituitary gland, yet reduced activity in other tissues, such as the prostate. Testosterone is metabolised differently in different tissues. While testosterone itself is the active agent in skeletal muscle, aromatisation to oestradiol is important in peripheral tissues, such as bone, liver, brain and adipose, and 5 α -reduction to DHT is important in the prostate. One such androgen is 7 α -methyl-19-nortestosterone (MENT). MENT is ten times more potent than testosterone in suppressing gonadotrophins and is resistant to 5 α -reduction [98] whilst it is aromatisable to an oestrogen [99]. It would therefore have the theoretical advantage of being relatively prostate sparing, which has in fact been demonstrated in castrated monkeys [100]. To date, the contraceptive effect of MENT has only been assessed in non-human primates, although clinical studies are underway. In combination with GnRH agonist, MENT maintained azoospermia for 8 months [101]. MENT does not bind to SHBG, thus it is rapidly cleared from the circulation. MENT acetate implants have been developed and result in dose-dependent antigonadotrophic activity in healthy men [102] whilst maintaining mood and sexual behaviour in hypogonadal men to a similar degree as testosterone [103].

4. Expert opinion

Over the past decade, we have seen significant progress in the quest to find a suitable male hormonal contraceptive. The available evidence suggests that both men and women would welcome this development [104,105]. However, several problems remain with existing methods. The main aims for the future will focus on improving methods of androgen delivery, reducing impacts on extra-testicular metabolism and increasing efficacy in suppression of spermatogenesis. With advances in cellular and molecular biology, the development of selective

modulators of both androgen and progesterone receptors may help achieve this goal, leading to a method which specifically suppresses spermatogenesis whilst maintaining muscle and bone mass and having no adverse effects on the prostate or lipid metabolism. The existence of multiple receptors as demonstrated for oestrogen, ER α and ER β [106], may also be true for androgens but have not been identified. Recently, a group of molecules have been identified which have selectivity and specificity for the AR [107] and their further modification may lead to them displaying agonist, antagonist or partial effects [108]. This may not only be of great benefit in the area of male hormonal contraception, but also may have more widespread clinical applications in the treatment of hypogonadism, androgen-dependent malignancy and in the development of HRT in ageing men. Similarly, the development of specific, orally active progesterone receptor agonists may replace conventional progestogens as an adjuvant in male contraceptive strategies [109,110].

Further understanding of the process of spermatogenesis, from the level of germ cell proliferation through epididymal maturation, will diversify the potential targets for male contraception. Evidence suggests that current hormonal regimes act both at the level of germ cell proliferation and later spermiation [111-113]. Further understanding of the factors controlling germ cell proliferation, meiosis and apoptosis will provide insight to potential targets.

5. Conclusion

Significant progress has been made in the development of a male hormonal contraceptive, and the first industry-based studies are underway. Using testosterone alone resulted in sub-optimal efficacy in suppression of spermatogenesis, however, results from combination regimes with progestogens are promising. The development of novel approaches using GnRH antagonists, including orally active compounds, and selective steroid receptor modulators will no doubt enhance and diversify current approaches. With increased pharmaceutical interest, the 'male pill' may become a reality.

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Demonstration of progesterone receptor-mediated gonadotrophin suppression in the human male

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Summary

OBJECTIVE Synthetic gestogens in combination with testosterone have potential as a male hormonal contraceptive, predominantly acting by augmenting suppression of gonadotrophin secretion. Little is known, however, of the effects of gestogens in the male. Gestogens have affinity for both androgen and progesterone receptors but the relative contribution of action at these two receptors in gonadotrophin suppression remains unclear. In this study the effects of progesterone, with no significant androgen-receptor affinity are compared to desogestrel, a synthetic gestogen with relatively low affinity for the androgen receptor, on gonadotrophin secretion in normal men.

DESIGN Subjects received either 50 mg progesterone intramuscularly (i.m.) or 300 µg desogestrel orally daily for 7 days. Frequent blood sampling over 12 h was undertaken before and after drug administration. GnRH [100 µg intravenously (i.v.)] was administered 2 h before the end of the frequent sampling period.

SUBJECTS Twenty healthy men were randomly allocated to the two treatment groups.

RESULTS Both progesterone and desogestrel administration resulted in decreases in the concentration of both LH and FSH secretion, as well as testosterone. Analysis of the pulsatile nature of LH secretion indicated that both treatments reduced LH pulse amplitude, and that progesterone reduced LH pulse frequency. Progesterone, but not desogestrel, treatment also reduced the increase in LH secretion in response to GnRH.

CONCLUSIONS The effects of progesterone were at least as marked as those of a maximally effective dose of desogestrel. As progesterone has negligible affinity for the androgen receptor, these results are compatible with the suppressive effects of synthetic 19-norgestogens on gonadotrophin secretion in the male being mediated via the progesterone receptor, with its androgenicity contributing minimally to gonadotrophin suppression.

One of the most promising approaches to hormonal male contraception involves the administration of testosterone with a gestogen, acting predominantly by suppression of gonadotrophin secretion (Meriggiola & Bremner, 1997; Brady & Anderson, 2002). The addition of the second agent allows lower doses of each steroid to be given, thus avoiding side-effects from supra-physiological doses of testosterone while maintaining effective spermatogenic suppression. Although having additive effects on the suppression of gonadotrophins (Wu *et al.*, 1999), the underlying mechanisms and the physiological importance of progestogens in suppressing gonadotrophin secretion in the male remain largely unknown. The synthetic gestogens, in particular the 19-norgestogens, have affinity for both androgen and progesterone receptors but the relative contribution of these two components in suppressing gonadotrophins is unclear.

In animal models, investigation of the effect of progesterone on gonadotrophin secretion in the male has given conflicting results. Following a large single injection of progesterone in adult rams, plasma LH levels were suppressed (Bolt, 1971). However, in other studies using infusions (Edgerton & Baile, 1977) or implants (Echternkamp & Lunstra, 1984; Van Lier *et al.*, 1999) no effect was reported. Recently, progesterone administration was found to suppress LH in castrate rams when given with testosterone (Turner *et al.*, 2001). The male progesterone receptor knock-out mouse demonstrates elevated circulating LH concentrations (Schneider *et al.*, 1999), suggesting a functional role for the progesterone receptor in the control of LH secretion.

Desogestrel is a synthetic third-generation 19-nortestosterone derivative. The active metabolite, 3-ketodesogestrel, demonstrates relatively high progesterone receptor selectivity in comparison to other synthetic gestogens such as gestodene and levonorgestrel (Phillips *et al.*, 1990). It has been a valuable component of the female combined oral contraceptive pill for many years, demonstrating desirable progestational activity with low

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androgenic effect. Recent studies of desogestrel in combination with testosterone esters as a hormonal contraceptive in men have had promising results, with azoospermia induced in 90–100% of subjects (Wu *et al.*, 1999; Anawalt *et al.*, 2000; Martin *et al.*, 2000; Kinniburgh *et al.*, 2002).

In this study we aimed to compare the effects of desogestrel with naturally occurring progesterone on LH and FSH secretion in healthy men. To date, there have been no studies in men comparing the effect of progesterone with desogestrel on the secretion of gonadotrophins. Progesterone, the natural steroid, has very low affinity for the androgen receptor, whereas desogestrel, although comparatively more potent and of greater selectivity than most of the synthetic gestogens, binds to both the progesterone and androgen receptors. We therefore hypothesized that if progesterone and desogestrel suppressed gonadotrophins to a similar extent it was unlikely that the suppressive effects of synthetic gestogens in the male are largely due to their androgenic properties.

Materials and methods

Subjects

Twenty normal men, aged 18–38, were recruited to participate in this study. They had a normal medical history and examination and haematological and biochemical screening obtained within 2 weeks of the start of the study. They were receiving no medication. Serum testosterone, FSH, LH and inhibin B concentrations, determined between 08:00 and 11:00 h were normal.

Experimental protocol

The study protocol was reviewed by the Lothian Research Ethics Committee and informed consent obtained. The study design was a single-centre open parallel-group study. Subjects were admitted to the clinical research area for a control period of frequent blood sampling, carried out at 15-min intervals via an indwelling catheter from 08:00 until 20:00 h. Two hours before the end of sampling, subjects were administered 100 µg GnRH intravenously (i.v.). Subjects were semiambulant throughout and did not fast. Subjects were then randomized by blocks of consecutive subjects to receive either desogestrel 300 µg orally daily or progesterone 50 mg intramuscularly (i.m.) daily for 7 days from the day following the first frequent sampling with the final dose administered on the morning of the second frequent blood sampling day. Two weeks after the second frequent sampling period, subjects attended for a post-treatment visit noting any adverse events.

Drugs

Desogestrel (NV Organon, Oss, the Netherlands) was taken at a dose of 300 µg (2 × 150 µg tablets) orally daily. Progesterone

(Ferring Pharmaceuticals Ltd, Langley, UK) 50 mg in 1 ml i.m. was administered daily by the investigator. GnRH (Fertalil, Hoechst Marion Rousell Ltd, Tokyo, Japan) was administered at a dose of 100 µg i.v. to each subject after 10-h frequent blood sampling which was then continued for a further 2 h.

Hormone assays

Blood was immediately centrifuged, plasma separated and stored at –20 °C until assayed. All samples were assayed for LH, and hourly samples for FSH, testosterone and inhibin B. Hormone assays were carried out as previously described (Martin *et al.*, 2000). Testosterone was measured by radioimmunoassay (RIA; Corker & Davidson, 1978) with a detection limit of 0.2 nmol/l and intra-assay variability of 8.9%. Plasma LH and FSH were measured by time-resolved immunofluorometric (in-house) assay and by highly sensitive immunoradiometric assay, respectively (NETRIA, St Bartholomew's Hospital, London, UK). The assay sensitivity was 0.15 IU/l (LH) and 0.1 IU/l (FSH) and intra-assay coefficients of variation 9% (LH) and 8% (FSH). Inhibin B was measured as previously described (Groome *et al.*, 1996). The detection limit of the assay was 15 pg/ml with an intra-assay coefficient of variation of 10.5%. Progesterone was measured using DPC Coat-A-Count Radioimmunoassay kit (DPC Biemann GmbH Diagnostika, Bad Nauheim, Germany), with a sensitivity of 0.06 nmol/l.

Data analysis

Mean hormone levels were calculated over 10 h of frequent blood sampling prior to the administration of GnRH. Pulses were identified by a modified version of that described by Santen & Bardin (1973) defining a pulse as an increment of greater than 20% (2.5 times intra-assay CV) sustained over two time points from the mean of the two values at the pulse nadir. Mean pulse frequency was then calculated over 10 h prior to the administration of GnRH and pulse amplitude from the peak to nadir difference (IU/l). The response to GnRH was calculated from the mean hormone values following the administration of GnRH over the final 2 h of frequent blood sampling. An alternative method, calculating the absolute difference from the peak and nadir concentrations, gave similar results. Results are presented as mean ± SEM. Hormonal data were analysed using Student's *t*-tests, paired when appropriate, on log-transformed data to correct heterogeneity of variance.

Results

Baseline characteristics

There were no significant differences between groups with regards to age, body mass index and pretreatment testosterone

and LH concentrations. While screening FSH concentrations were significantly higher in the desogestrel group (Table 1), the analysis of mean control FSH levels (pre-GnRH administration) revealed no significant difference between groups. Serum progesterone concentrations at 0.75 h and 9.25 h of frequent blood sampling following 7 days of progesterone administration were 113.2 ± 14.4 nmol/l and 116.6 ± 8.2 nmol/l, respectively.

Mean hormone concentrations

Both desogestrel and progesterone treatment resulted in significant falls in the mean concentrations of LH and FSH (*P* < 0.005, Table 2) and of testosterone (*P* < 0.005). There was no significant change in serum inhibin B with either treatment.

Table 1 Baseline characteristics of subjects

Parameter	Progesterone	Desogestrel
Age (years)	24.2 ± 1.5	25.4 ± 1.5
BMI (kg/m ²)	23.8 ± 0.9	24.3 ± 1.0
Testosterone (nmol/l)	19.5 ± 2.6	21.4 ± 2.6
LH (IU/l)	4.5 ± 0.9	4.4 ± 0.9
FSH (IU/l)	3.1 ± 0.2	4.3 ± 1.0*

Data are presented as mean ± SEM, **P* < 0.05 vs. progesterone group.

Pulsatile LH secretion

LH pulse amplitude was similar in the two groups pretreatment (Fig. 1), although the mean number of pretreatment pulses in 10 h was lower in the desogestrel group than in the progesterone group (*P* = 0.03). There was a significant decline in pulse amplitude with both treatments, from 2.4 ± 0.5 to 1.6 ± 0.4 IU/l (*P* < 0.05) in the progesterone-treated group and from 1.9 ± 0.2 to 1.3 ± 0.3 IU/l (*P* < 0.05) in the desogestrel-treatment group (Fig. 1). There was also a significant decline in the frequency of LH pulses following progesterone treatment, from 4.7 ± 0.4 to 3.8 ± 0.4/10 h (*P* < 0.005; Figs 1 and 2a). Desogestrel treatment resulted in a nonsignificant decrease in LH pulse frequency, from 3.5 ± 0.2 to 3.0 ± 0.3/10 h (*P* = 0.3; Figs 1b and 2).

Gonadotrophin response to GnRH

All men showed brisk increases in both LH and FSH secretion in response to GnRH administration. There was a significant attenuation of the response to GnRH in the progesterone-treated group. Mean post-GnRH LH concentration fell from 16.3 ± 4.3 to 10.5 ± 2.6 IU/l (*P* < 0.0005), and FSH fell from 3.6 ± 0.3 to 2.0 ± 0.2 IU/l (*P* < 0.005; Fig. 3). In the desogestrel-treated group there was a significant fall in FSH from 4.5 ± 0.5 to

Parameter	Progesterone		Desogestrel	
	Control	Treatment	Control	Treatment
Testosterone (nmol/l)	21.68 ± 1.94	8.75 ± 0.68*	21.58 ± 2.87	8.03 ± 2.09*
LH (IU/l)	2.92 ± 0.71	1.57 ± 0.32*	2.58 ± 0.39	1.42 ± 0.36*
FSH (IU/l)	2.14 ± 0.22	1.09 ± 0.15*	2.88 ± 0.34	1.48 ± 0.20*
Inhibin (IU/l)	211.8 ± 36.6	186.9 ± 19.7	219.6 ± 35.0	229.7 ± 30.4

Data are presented as mean ± SEM, **P* < 0.005 vs. control. Means are average of all data over 10 h pre-GnRH administration.

Table 2 Mean hormone concentrations (pre-GnRH administration)

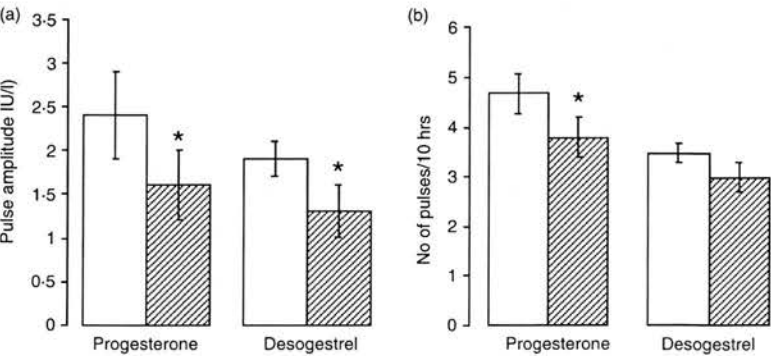


Fig. 1 (a) Mean LH pulse amplitude (IU/l), control and following 7 days treatment with i.m. progesterone or oral desogestrel as indicated, **P* < 0.05 vs. control. (b) Mean LH pulse frequency (pulses/10 h), control (open columns) and following 7 days treatment (hatched columns) with i.m. progesterone and oral desogestrel as indicated, *P* < 0.005 vs. control.

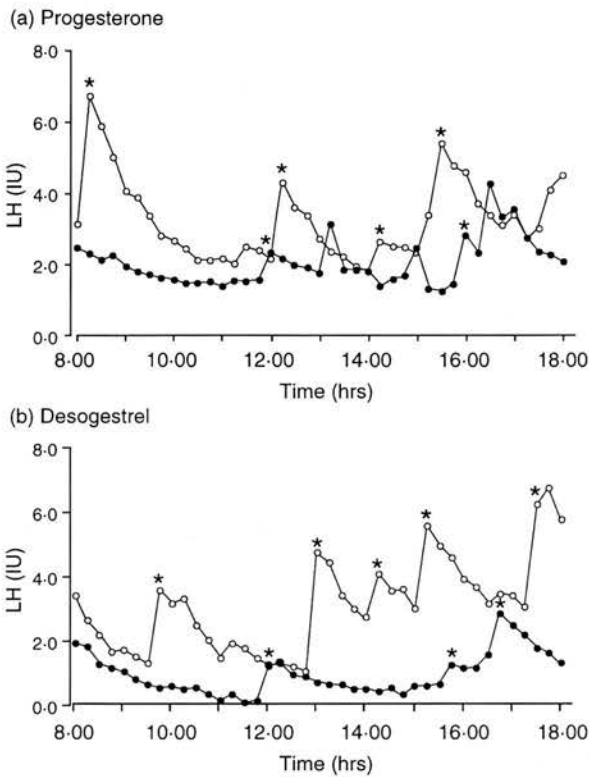


Fig. 2 Pulsatile LH secretion pre-GnRH administration in representative individual subjects from (a) progesterone and (b) desogestrel groups. Control (open circles) and following 7 days treatment (closed circles), *denotes pulse.

2.8 ± 0.4 ($P = 0.04$), and a nonsignificant fall in LH from 9.5 ± 0.9 to 7.3 ± 1.0 IU/l ($P = 0.13$; Fig. 3).

Discussion

These results demonstrate the antigonadotrophic action of progesterone and desogestrel in healthy men. A dose of 300 µg was chosen for desogestrel as this has been demonstrated to be optimal in male contraceptive regimes giving mean serum levels of > 1000 pg/ml (Wu *et al.*, 1999) with no added benefit from a further increase in dose to 450 µg (Bellis *et al.*, 1996). Progesterone concentrations achieved were in the upper physiological range of the luteal phase in women. Both progesterone, the natural steroid, and synthetic desogestrel significantly decreased LH and FSH concentrations and the secretion of testosterone. Both steroid treatments reduced the pulse amplitude of LH secretion, and progesterone also significantly reduced LH pulse frequency. As our model does not allow dissection of the hypothalamic-pituitary axis, these results do not allow us to determine their level of action.

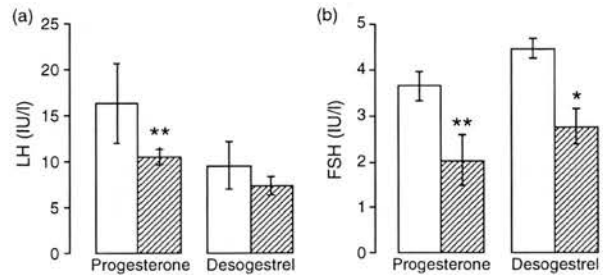


Fig. 3 Mean (a) LH and (b) FSH response following GnRH administration after 10 h of frequent blood sampling. Control (open columns) and following 7 days treatment (hatched columns) with im progesterone or oral desogestrel as indicated. * $P < 0.05$ and ** $P < 0.005$ vs. control.

In common with other steroids, gestogens bind to a variety of receptors with differing affinity. Receptor-binding studies have compared the relative binding affinities of both progesterone and desogestrel (Pollow *et al.*, 1989; Phillips *et al.*, 1990). Although desogestrel has a high selectivity index for the progesterone receptor in comparison to older synthetic gestogens, it still has significant affinity for the androgen receptor while the natural steroid demonstrates negligible binding. In view of the relative binding affinities of both steroids for the androgen and progesterone receptor, these results suggest that the antigonadotrophic effect of 19-norgestogens including desogestrel is unlikely to be mediated solely due to their androgenicity. We acknowledge, however, that these results do not positively demonstrate that the suppression of gonadotrophin secretion by desogestrel is mediated by the progesterone receptor.

Previous studies of steroidal feedback on the hypothalamic-pituitary axis in the male have focussed on the effects of exogenous androgens (Matsumoto & Bremner, 1984; Finkelstein *et al.*, 1991; Hayes *et al.*, 2001) and, more recently, oestrogens (Finkelstein *et al.*, 1991; Handelsman *et al.*, 2000; Hayes *et al.*, 2000), with relatively little data regarding the effects of progesterogens. In the female, the antigonadotrophic effects of synthetic gestogens have been investigated (Couzinet *et al.*, 1996) and shown to be mediated through the progesterone receptor: the suppressive effects of synthetic gestogens on LH and FSH were not reversed on selectively blocking the androgen receptor with flutamide and their antigonadotrophic action was similar in both healthy subjects and those with complete androgen insensitivity.

Consistent with other studies in normal men (Bellis *et al.*, 1996; Wu *et al.*, 1999; Anawalt *et al.*, 2000; Kinniburgh *et al.*, 2002), desogestrel treatment resulted in a significant decline in both LH and FSH secretion. The significance of the fall in LH is evident in the concomitant decrease in testosterone concentrations. Despite this decreased endogenous gonadal steroid feedback, both treatments still resulted in suppression of LH and FSH secretion. Indeed, the degree of suppression of gonadotrophins

is likely to have been yet greater if testosterone levels had been maintained by exogenous administration (Wu *et al.*, 1999). As expected from longer-term male hormonal contraceptive studies (Martin *et al.*, 2000; Kinniburgh *et al.*, 2002), and despite the fall in FSH secretion, serum inhibin B concentrations were unchanged after a week of treatment in either group. Consequently, the decline in FSH cannot be attributed to any change in feedback inhibition from inhibin B.

It is difficult to ascertain at what level gonadal steroids and their synthetic derivatives exert their inhibitory effect on gonadotrophin secretion, as this is dependent on the integrated response of both the hypothalamus and pituitary. In this study, we have used two means of indirectly assessing GnRH secretion. First, peripheral LH was used as a surrogate marker for GnRH. Portal catheterization models in the sheep have demonstrated that the frequency of pulsatile LH secretion is closely synchronized to hypothalamic LHRH secretion (Clarke & Cummins, 1982). The frequency of pulsatile LH secretion pretreatment in the present study is similar to previously published data (Crowley *et al.*, 1985) and differences were detected in both desogestrel and progesterone groups. It is possible that more frequent sampling intervals over a longer period would have improved pulse detection in the desogestrel group; however, the low control baseline may have contributed to this result. The second method is through the measurement of LH pulse amplitude. This, however, is more complex; in addition to reflecting the quantity of GnRH release it is also influenced by the effect of endogenous gonadal steroids on the sensitivity of the pituitary. A hypothalamic site of action may be suggested by the significant decline in LH pulse frequency in the progesterone-treated group (Figs 1 and 2). Indeed, progesterone receptors have been identified in the hypothalamus of nonprimate (Lauber *et al.*, 1991; Turner *et al.*, 2001) and primate (Betha *et al.*, 1992) animal species. Interestingly, the suppressive effect of progesterone in the ram was reduced following castration (Turner *et al.*, 2001), perhaps suggesting an interaction between progesterone receptor expression and that of other gonadal steroids, as in the endometrium. There are no data localizing progesterone receptors in the human hypothalamus. The reason for the smaller, nonsignificant, decline in the desogestrel group is unclear but may be related to the slightly lower basal levels and frequency of LH pulses in this group. While the variance was higher in the progesterone group, the same result was determined using both parametric and non-parametric testing. It is unlikely that we would have seen a greater effect with a higher dose of desogestrel (Bellis *et al.*, 1996).

We also examined the pituitary reserve by giving a bolus of GnRH at a maximal dose. However, we cannot assume that the response to exogenous GnRH approximates that of endogenous GnRH. Conversely, any effect at the level of the pituitary cannot be presumed to result from a direct effect of the administered steroid as it may be secondary to altered endogenous GnRH

secretion. A significant attenuation of the pituitary response (both LH and FSH) to GnRH was observed in the progesterone-treated group. However in the desogestrel-treated group, a significant attenuation of only the FSH response was seen. Without a 'hypothalamic clamping' model we cannot conclude that this is a direct pituitary effect of treatment. Progesterone receptors have been localized by immunohistochemistry to the gonadotroph of the human female pituitary gland (Couzinet *et al.*, 1999). However, a sex-related difference in the distribution of the progesterone receptor is evident in the rat (Rainbow *et al.*, 1982), which may be also true of humans and there are no data at present directly demonstrating the presence of the progesterone receptor in the human male pituitary gland.

One possible mechanism whereby progesterone and synthetic gestogens may alter gonadotroph function is through decreasing the number of GnRH receptors in the pituitary. This is supported by evidence both *in vitro* and *in vivo*. In the orchidectomized sheep, progesterone stimulation reduces the concentrations of the GnRH receptor and GnRH receptor mRNA in pituitary tissue (Sakurai *et al.*, 1997). Similarly, progestin-induced falls in concentrations of GnRH receptor and receptor mRNA have been noted in ovine pituitary cells in culture (Phillips *et al.*, 1988; Laws *et al.*, 1990; Wu *et al.*, 1994). Progesterone may regulate gonadotrophin synthesis at both transcriptional and post-transcriptional levels. The 5' flanking region of the ovine gonadotropin FSH- β gene has several progestin response elements (Webster *et al.*, 1995) and progesterone has been reported to shorten the length of the poly(A) tail of mRNA encoding gonadotrophin subunits (Wu & Miller, 1991). Therefore, progesterone may affect the steady-state concentration of gonadotrophin mRNA by altering both the rate of gene transcription and the degree of mRNA stability. This may be the mechanism for the clear suppression of FSH that is evident with both progesterone and desogestrel. Although 3-keto-desogestrel exhibits weak glucocorticoid activity, it is unlikely that glucocorticoid receptor-mediated activity could account for the antigonadotrophic effects observed. Progesterone has low affinity and desogestrel has weak antagonistic effect at the glucocorticoid receptor (Fuhrmann *et al.*, 1995). Furthermore, it is only chronic glucocorticoid excess that has been associated with impairment of the hypothalamic-pituitary axis (MacAdams *et al.*, 1986).

In conclusion, these data demonstrate progesterone receptor-mediated suppression of gonadotrophin secretion in normal men. The demonstration that progesterone had comparable effects on gonadotrophin secretion to desogestrel indicates that synthetic gestogens exert their antigonadotrophic effect through their progestogenic properties. We have not examined the relative contribution of their androgenicity. The level of interaction at the hypothalamic-pituitary axis remains unclear, although both may be involved. These data are of importance in determining the mechanism of action of agents used in male contraception. The

further elucidation of their mechanism of action may enable development of regimes with increased tissue selectivity and thus greater specificity of action with fewer side-effects.

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Depot testosterone with etonogestrel implants result in induction of azoospermia in all men for long-term contraception

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BACKGROUND: Combined testosterone and progestogen preparations are a promising approach to male hormonal contraception. We investigated the effect of s.c. etonogestrel with depot testosterone on spermatogenesis in normal men over a period of 48 weeks. **METHODS:** Fifteen healthy men received three s.c. 68 mg etonogestrel implants. Testosterone pellets (400 mg) were administered at 12 weekly intervals. **RESULTS:** Nine men completed 48 weeks of treatment. Four subjects chose to discontinue after 6 months, one man withdrew from the study early for personal reasons and one was withdrawn due to illness. Sperm concentrations of $<1 \times 10^6/\text{ml}$ were achieved in all men by 16 weeks of treatment. All men became azoospermic, although the time to achieve this varied from 8 to 28 weeks. Azoospermia was maintained in eight of the nine men treated for 48 weeks, one subject showing partial recovery from 40 weeks. Testosterone levels remained in the physiological range throughout. Treatment did not result in weight gain, change in body composition or decline in high-density lipoprotein cholesterol concentrations. **CONCLUSIONS:** The combination of three etonogestrel implants with depot testosterone results in rapid and consistent suppression of spermatogenesis. This can be maintained for up to 1 year and may therefore be a suitable approach for a long-acting male hormonal contraceptive.

Key words: etonogestrel/male contraception/progestogen/spermatogenesis/testosterone

Introduction

Current approaches to male hormonal contraception are based on the withdrawal of the gonadotrophin support to the testis resulting in suppression of spermatogenesis and intra-testicular testosterone (Anderson and Baird, 2002; Nieschlag *et al.*, 2003). Two large international studies sponsored by the World Health Organization (WHO) administering high dose testosterone enanthate demonstrated that hormonal suppression of spermatogenesis sufficient for contraceptive efficacy is a possibility (World Health Organization Task Force on Methods for the Regulation of Male Fertility, 1990, 1996). However, such regimes resulted in metabolic side effects such as a fall in high-density lipoprotein cholesterol (HDL-C) concentration, acne and weight gain due to supra-physiological testosterone levels (Wu *et al.*, 1996), and revealed ethnic polymorphism in spermatogenic response, with a lesser suppression among Caucasians than in Asians. Progestogens are effective suppressors of gonadotrophin secretion, but the concomitant fall in testosterone production by the testis necessitates the co-administration of androgen. The introduction of a progestogen acting synergistically with testosterone allows a lowering of the total testosterone dose

avoiding unwanted metabolic effects, and may also enhance the degree of spermatogenic suppression, potentially by direct intra-testicular effects (Bebb *et al.*, 1996; Meriggiola *et al.*, 1996; McLachlan *et al.*, 2002; Zhang *et al.*, 2003). Several progestogens have been investigated in this context, including medroxyprogesterone acetate (Knuth *et al.*, 1989; World Health Organization Task Force on Methods for the Regulation of Male Fertility, 1993; Handelsman *et al.*, 1996; Turner *et al.*, 2003), levonorgestrel (Bebb *et al.*, 1996), cyproterone acetate (Meriggiola *et al.*, 1996, 1998) and norethisterone enanthate (Kamischke *et al.*, 2001). Promising results have been obtained using oral desogestrel, with high rates of azoospermia achieved in men from several ethnic backgrounds (Wu *et al.*, 1999; Anawalt *et al.*, 2000; Kinniburgh *et al.*, 2002).

Compared with oral administration, a long-acting drug delivery system has advantages, including dose-sparing and the avoidance of hepatic exposure to high doses, both of which may contribute to the reduction of unwanted adverse effects. Moreover, it may be preferred by some individuals because of ease of compliance (Martin *et al.*, 2000a). Etonogestrel, the active metabolite of oral desogestrel, has

been marketed recently in many countries as a long-acting implant (Implanon[®], NV Organon, Oss, The Netherlands) providing 3 years of contraceptive efficacy in women. We have reported previously our experience with one or two etonogestrel implants in combination with depot testosterone pellets (Anderson *et al.*, 2002). Although profound suppression of spermatogenesis with minimal non-reproductive side effects was induced, azoospermia was achieved in only 64 and 75% of the one and two implant groups, respectively. Etonogestrel implants release $\sim 50 \mu\text{g/day}$, thus even with two implants the daily dose is markedly lower than the optimally effective dose of $300 \mu\text{g}$ desogestrel, which has $\sim 80\%$ oral bioavailability (Hasenack *et al.*, 1986). There was therefore evidence for significant dose-sparing with the implant preparation but, as spermatogenic suppression was not complete in all men, we hypothesized that the addition of a third etonogestrel implant may enhance this spermatogenic suppression. In this study, we additionally have extended the duration of treatment to 48 weeks to investigate whether the steady decline in etonogestrel release from the implants will maintain suppression of gonadotrophins and thus spermatogenesis for that length of time, using the same testosterone regimen we have used previously in the investigation of both oral desogestrel and etonogestrel implants.

Methods

Subjects

Fifteen healthy men (mean age 31.6 years, range 18–37) were recruited from the same general population as previous studies (Anderson *et al.*, 2002; Kinniburgh *et al.*, 2002). Inclusion criteria included age (18–45), mentally and physically healthy, body mass index (BMI) between 18 and 32 kg/m^2 , normal pre-treatment FSH, LH and testosterone concentrations, routine haematological and biochemical analyses, two normal semen analyses according to WHO criteria at least 2 weeks apart, and a normal physical and andrological examination. Pre-treatment sperm concentrations were $> 20 \times 10^6/\text{ml}$ in all men, and motility and morphology were within normal ranges for the local population. Subjects provided written informed consent and the study had ethical approval from Lothian Reproductive Medicine Ethical Review Committee. The study was performed according to GCP guidelines.

Study design and medication

This study was a single-group open investigation of the effects of etonogestrel implants with testosterone pellets. The duration of the treatment period was 48 weeks, with those subjects who were not azoospermic discontinuing treatment if they wished at 24 weeks. Following pre-treatment assessment, three implants each containing 68 mg etonogestrel (Implanon, NV Organon, Oss, The Netherlands) were inserted s.c. in the medial aspect of the non-dominant upper arm to all subjects. All subjects additionally received 400 mg testosterone pellets ($2 \times 200 \text{ mg}$, NV Organon) inserted s.c. under local anaesthetic into the anterior abdominal wall on the day of insertion of the etonogestrel implants, and 12 weekly thereafter for the duration of the treatment period, i.e. at 12, 24 and 36 weeks.

During treatment and recovery, subjects attended at 4 weekly intervals for medical review, and for semen analysis and venesection. Additional blood samples were drawn pre-treatment and at weeks 4 and 12 between 07.30 and 09.30 (a.m. samples) and

between 16.30 and 18.30 (p.m. samples) for testosterone measurement. Subjects were examined at weeks 12, 24, 36, 48 and at final visits, and a morning first-void urine sample was obtained at the same time points for measurement of epitestosterone. Bio-electrical impedance was determined as described (Davies and Preece, 1988; Gregory *et al.*, 1991) using the Holtain Body Composition Analyser (Holtain Ltd, Dyfed, UK) and fat-free mass and percentage body fat determined for each subject at screening, 12 weekly thereafter and at 16 weeks of the recovery period. Throughout the study, any adverse events were noted at each visit. During the recovery phase, subjects attended at 4 weekly periods for a minimum of 16 weeks up to 24 weeks until semen analysis returned to normal by WHO criteria. Subjects with semen analysis below normal WHO criteria were followed-up beyond this period until normal values were attained.

Assays

Blood samples were obtained in fasting subjects (for glucose and lipids) and plasma separated by centrifugation at 4000 g for 15 min and stored at -20°C until hormone assay. Testosterone was measured by radioimmunoassay (Corker and Davidson, 1978), and LH, FSH and sex hormone-binding globulin (SHBG) by a time-resolved immunofluorometric in-house assay. Assay sensitivity was 0.3 nmol/l for testosterone, 0.5 nmol/l for SHBG, 0.03 IU/l for FSH and 0.15 IU/l for LH. The intra-assay coefficients of variation (CVs) were $< 10\%$ for testosterone, FSH and LH, and 4% for SHBG. The inter-assay CVs were 12.4% for testosterone, $< 10\%$ for FSH and LH, and 8.8% for SHBG. Free testosterone was calculated as described (Vermeulen *et al.*, 1999). Urinary epitestosterone concentrations (aglycone plus free fraction) were determined by gas chromatography–mass spectrometry as described and validated previously (Kicman *et al.*, 1995; Coutts *et al.*, 1997). Between-assay precision was $< 8\%$ for epitestosterone concentrations between 27 and 133 nmol/l , and 13.4% at 5 nmol/l . The assay sensitivity was 0.87 nmol/l . Inhibin B was measured in both serum and seminal plasma by methods previously described (Groome *et al.*, 1996; Anderson *et al.*, 1998) with an assay sensitivity of 7.8 pg/ml . Etonogestrel was measured by in-house radioimmunoassay by Organon NV, assay sensitivity 30 pg/ml . Intra-assay CV was 9% and inter assay CV was 14% . Samples were analysed for general haematological and biochemical values (including total cholesterol and HDL-C) by routine autoanalyser at 12 weekly intervals.

Semen analysis

At all assessments, semen analysis was carried out using WHO methodology (World Health Organization, 1999). Local normal values for motility are $> 27\%$ grade a + b, or $> 36\%$ grade a + b + c and normal morphology $> 15\%$. Azoospermia was confirmed following centrifugation of the whole semen sample. Centrifugation was performed at 3660 g for 15 min, and a sample was classified as azoospermic only after a systematic examination of the re-suspended precipitate indicated the complete absence of spermatozoa.

Behavioural assessment

Sexual activity and interest were investigated by means of a structured questionnaire used to quantify sexual activity over the preceding 2 week period (Anderson *et al.*, 1992). This was carried out before treatment and at 12 weekly intervals thereafter.

Statistical analysis

Results are presented as mean \pm SEM. Hormone data were log transformed and semen concentrations cube root transformed before

analysis by ANOVA (analysis of variance) for repeated measures. Paired *t*-tests were used to investigate at what time points significant treatment effects were evident, with the exception of behavioural data which were analysed using the Wilcoxon matched pair test for non-parametric testing. For all comparisons, a *P*-value of <0.05 was considered significant.

Results

Subjects, adverse events and withdrawals

Of the 15 men entering the study, nine completed 48 weeks of treatment. Four chose to leave the study after 24 weeks for personal reasons. One man was withdrawn from the study at 24 weeks due to inter-current illness (acute alcohol toxicity). One man withdrew from the study for personal reasons after 4 weeks treatment; thus data from this individual are not included in the analysis. Adverse events experienced included low mood (three subjects) and testosterone pellet extrusion (two subjects, replacement pellets administered), but none resulted in any subject withdrawing from the study. Removal of etonogestrel implants was uncomplicated in all men. Pre-treatment data are presented in Table I.

Sperm concentrations

There was a profound suppression of spermatogenesis during the study (Figure 1), and all 14 men became azoospermic eventually. After 16 weeks of treatment, sperm concentration in all subjects was below the threshold of $1 \times 10^6/\text{ml}$, with 10 of 14 subjects (71%) azoospermic (Figure 1b). At 24 weeks, 11 men were azoospermic, and sperm concentrations were $<0.1 \times 10^6/\text{ml}$ in the other three. These three were among the nine subjects who continued the study for the full 48 weeks, and all were azoospermic at 28 weeks. The range of time to azoospermia was 8–28 weeks, median 16 weeks. Eight men remained azoospermic until the end of the 48 week treatment period. One man showed partial recovery of spermatogenesis, with spermatozoa detectable at week 40 ($0.7 \times 10^6/\text{ml}$) and sperm concentration increasing to $7 \times 10^6/\text{ml}$ at 48 weeks.

During the recovery phase, 60% of subjects had reached sperm concentrations in the normal range by week 16, and 79% by week 24. Incomplete follow-up data were obtained in the subject who was discontinued from the study due to inter-current illness and in one other man. The remaining two subjects were followed-up until normal sperm concentrations were demonstrated at 32 and 48 weeks after implant removal.

Table I. Pre-treatment values for subjects included in the study	
	<i>n</i> = 15
Age (years)	31.6 ± 1.3 (range 18–38)
BMI (kg/m ²)	25.8 ± 0.9 (range 20.5–31.9)
LH (IU/l)	4.0 ± 0.4
FSH (IU/l)	5.3 ± 0.8
Testosterone (nmol/litre)	22.1 ± 1.7

Data are presented as the mean ± SEM.

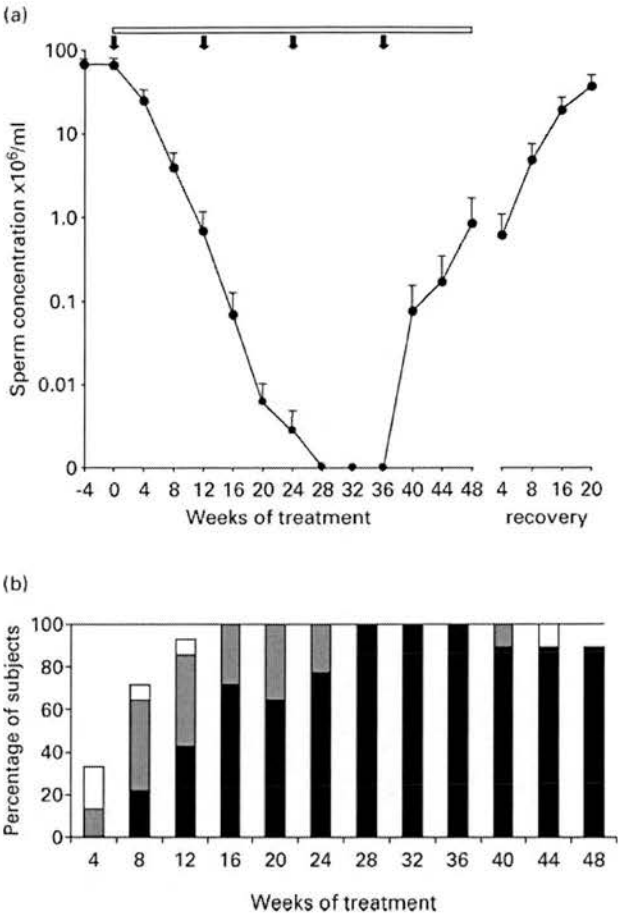


Figure 1. (a) Sperm concentrations during etonogestrel/testosterone treatment and the recovery period. Duration of treatment is indicated by the bars and the time points of testosterone implant insertion are indicated by arrows. Note the log scale on the ordinate. Data are presented as mean ± SEM, *n* = 14 for the first 24 weeks; thereafter nine men continued for 48 weeks. (b) Percentage of men achieving azoospermia (■), and concentrations of $<1 \times 10^6/\text{ml}$ (■) and $1 < 3 \times 10^6/\text{ml}$ (□) at each time point during treatment.

Testosterone and epitestosterone concentrations

Serum testosterone concentrations remained within the normal physiological range throughout the treatment period, with fluctuations according to the timing of testosterone pellet re-administration (Figure 2a). A gradual decline was observed from pre-treatment values reaching statistical significance at week 4 (*P* = 0.0006) with a nadir at week 12. Following re-administration of testosterone at week 12, concentrations rose to levels that were not significantly different from baseline at week 16, with a similar pattern of fluctuation throughout the remainder of the treatment period. During the recovery phase, testosterone concentrations rapidly returned to pre-treatment concentrations. Calculated free testosterone concentrations showed a similar pattern, with nadir concentrations significantly lower than pre-treatment (*P* < 0.01, Table II) and returning to pre-treatment levels during the recovery phase. During the treatment phase, free testosterone concentrations showed a gradual

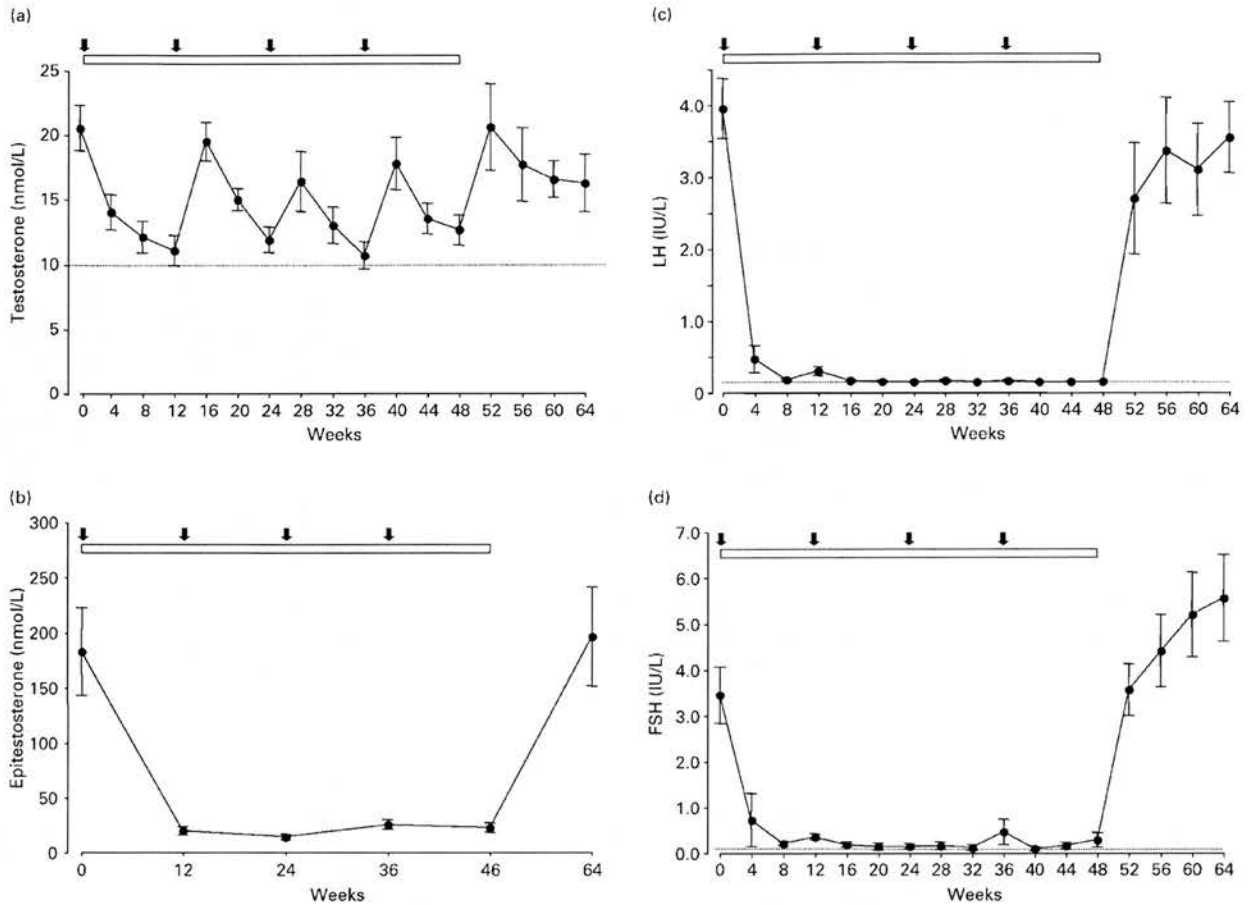


Figure 2. (a) Testosterone, (b) epitestosterone, (c) LH and (d) FSH concentrations during etonogestrel and testosterone treatment. The treatment period is indicated by the bars, with time points of testosterone pellet insertion indicated by arrows. Data are presented as the mean \pm SEM. In (a), the broken line indicates the lower limit of the normal range. In (c and d), the lower limits of detection of the assays are indicated by broken lines; in (b), the limit of detection is 0.89 nmol/L.

rise from week 12 (0.30 ± 0.03 nmol/L) to week 48 (0.39 ± 0.03 nmol/L), which was not statistically significant.

Urinary epitestosterone concentrations were suppressed by week 12 ($P = 0.001$) to $\sim 10\%$ of pre-treatment concentrations (Figure 2b), remaining readily detectable in all samples. Epitestosterone concentrations remained consistently

suppressed throughout treatment without significant change, returning to pre-treatment concentrations by 12 weeks of recovery.

A diurnal variation in serum testosterone concentrations was observed pre-treatment (Figure 3), concentrations in the morning being an average of 35% higher than in the early

Table II. Haematological, lipid, SHBG and free testosterone concentrations pre-treatment and during treatment at the indicated time-points and after 16 weeks recovery

	Pre-treatment	12 weeks	24 weeks	36 weeks	48 weeks	Recovery
Haemoglobin (g/l)	152 ± 1.7	152 ± 2.6	154 ± 1.5	154 ± 2.4	$155 \pm 2.1^*$	$157 \pm 2.1^*$
Haematocrit	0.45 ± 0.01	0.44 ± 0.01	0.45 ± 0.01	0.45 ± 0.01	0.45 ± 0.01	0.46 ± 0.01
Cholesterol (mmol/l) ^a	5.3 ± 0.4	5.0 ± 0.4	$4.6 \pm 0.3^*$	4.9 ± 0.3	4.5 ± 0.3	5.3 ± 0.7
HDL-C (mmol/l)	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.3 ± 0.2
LDL-C (mmol/l)	3.7 ± 0.4	3.5 ± 0.4	3.2 ± 0.3	3.5 ± 0.3	3.3 ± 0.2	3.6 ± 0.4
Triglycerides (nmol/l) ^b	2.1 ± 0.3	1.7 ± 0.4	$1.6 \pm 0.2^*$	1.5 ± 0.3	$1.2 \pm 0.2^*$	1.9 ± 0.4
Free testosterone (nmol/l) ^c	0.52 ± 0.05	$0.30 \pm 0.03^*$	$0.34 \pm 0.02^*$	$0.33 \pm 0.03^*$	0.39 ± 0.03	0.44 ± 0.09
SHBG (nmol/l) ^d	25.6 ± 2.7	$18.2 \pm 1.6^*$	$15.5 \pm 1.4^*$	$12.2 \pm 1.9^*$	$13.2 \pm 2.2^*$	22.2 ± 2.9

Data are presented as the mean \pm SEM.

^aCholesterol concentrations were significantly lower at 24 weeks of treatment ($P = 0.006$).

^bTriglyceride concentration was significantly lower at week 24 ($P = 0.05$) and week 48 ($P = 0.02$) of treatment.

^cFree testosterone concentrations were significantly lower from week 12 ($P = 0.003$) until week 36.

^dSHBG concentrations were significantly lower from week 12 ($P = 0.001$) and remained significantly decreased until recovery.

*Further significant ($P < 0.05$) treatment changes following ANOVA.

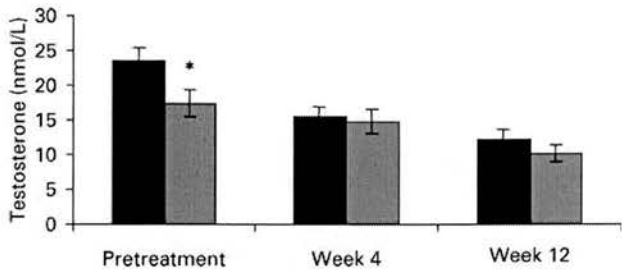


Figure 3. Mean a.m. ■ and p.m. ■ serum testosterone concentrations (nmol/l) in subjects ($n = 11$) receiving etonogestrel and testosterone treatment at pre-treatment and after 4 and 12 weeks treatment. Data are presented as mean \pm SEM. * $P < 0.05$ versus the a.m. sample.

evening ($P = 0.002$). After 4 weeks of treatment, this was lost, with no significant differences between morning and evening concentrations. Concentrations at both times of day at 4 weeks, however, were not significantly different from pre-treatment early evening concentrations. At 12 weeks of treatment, mean testosterone concentrations were low, this being immediately prior to re-administration of the testosterone pellets, but were again similar in the morning and evening. Comparison of the diurnal variation in testosterone concentrations between pre-treatment and 12 weeks showed a significant difference ($P < 0.05$).

Other reproductive hormones

Treatment with etonogestrel and testosterone resulted in profound suppression of both LH and FSH ($P < 0.0001$ versus pre-treatment from week 4 onwards). Some fluctuation in suppression was evident at 12 and 36 weeks (FSH) and 12 weeks (LH), at the times of trough testosterone concentrations (Figure 2c and d). During the later weeks of the study, LH was consistently suppressed to undetectable concentrations in all men at 24 weeks of treatment and for the rest of the treatment period in all men who continued to 48 weeks. Suppression of FSH was more variable, being detectable in up to two-thirds of subjects at time points of trough testosterone concentrations. More consistent partial recovery of FSH concentrations was seen in three men during the final 8 weeks of the study, particularly in the one individual who showed some restoration of spermatogenesis. In this individual, FSH during the second half of the treatment period was undetectable only at week 40, with a mean concentration between weeks 28 and 48 of 0.5 IU/l. Two further individuals with partial escape of FSH suppression (mean concentrations between weeks 28 and 48 of 0.1 and 0.8 IU/l) maintained azoospermia. Both gonadotrophins rapidly recovered following treatment. There was a progressive rise in FSH from weeks 4 to 16 of the recovery phase, at which time FSH concentrations were significantly higher than pre-treatment ($P = 0.02$).

Serum inhibin B concentrations showed a gradual decline over the course of treatment, continuing to week 48 ($P < 0.001$; Figure 4). This reached statistical significance from week 4 of treatment onwards ($P = 0.047$). By week 16

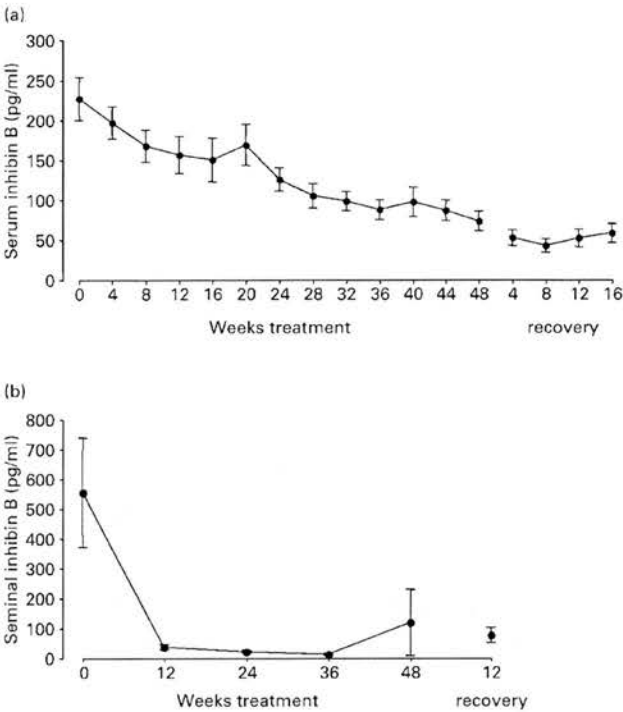


Figure 4. (a) Serum inhibin B and (b) seminal plasma inhibin B concentrations during etonogestrel and testosterone treatment. The treatment period is indicated by the bars, with the time points of testosterone implant insertion indicated by arrows. Data are presented as mean \pm SEM. The large error bar at 48 weeks (b) reflects partial recovery in one individual: seminal plasma inhibin B was undetectable in all other samples at that time.

of the recovery phase, serum inhibin B levels showed only limited evidence of recovery, remaining significantly lower than pre-treatment ($P < 0.001$).

Seminal plasma inhibin B concentrations were profoundly suppressed during treatment ($P = 0.02$ pre-treatment versus week 12). Seminal plasma inhibin B was undetectable in eight of 13 subjects by week 24. In the latter 24 weeks of the study, it was undetectable in all subjects except the individual who demonstrated recovery of spermatogenesis. This subject showed an increase in seminal plasma inhibin B at 36 weeks (having been at the limit of detection at week 24). This thus preceded detectable spermatogenic recovery, as at that time the subject was azoospermic but had a sperm concentration of $0.7 \times 10^6/\text{ml}$ 4 weeks later.

SHBG showed a gradual decline over the treatment period (Table II). This reached statistical significance by week 4 ($P = 0.0002$) and continued to week 48. During recovery, SHBG returned to pre-treatment concentrations.

Etonogestrel

Serum etonogestrel concentrations were highest 4 weeks after implant insertion, with a mean concentration of $765 \pm 57 \text{ pg/ml}$. Etonogestrel concentrations showed a gradual decline thereafter (Figure 5), being 63% of peak levels at 24 weeks and 43% at week 48. Etonogestrel was undetectable in all subjects 4 weeks after implant removal. The individual

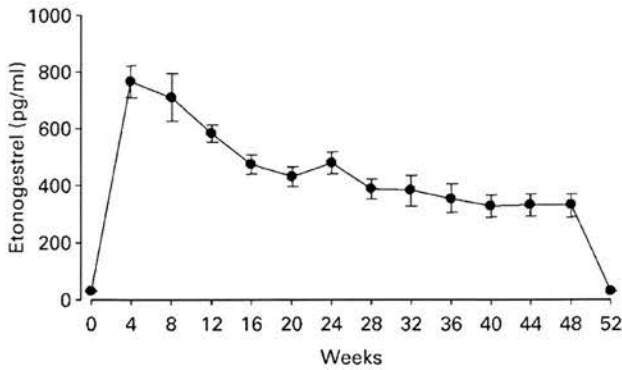


Figure 5. Etonogestrel concentrations during etonogestrel/testosterone treatment (0–48 weeks) and 4 weeks after removal of etonogestrel implants, at which time it was undetectable in all men. Data are presented as mean \pm SEM.

who showed partial spermatogenic recovery had serum etonogestrel concentrations close to the group mean.

Lipids and haematology

Cholesterol concentrations showed a gradual fall during etonogestrel and testosterone treatment, statistically significant at week 24 ($P = 0.006$) and returning to baseline during recovery (Table II). Similarly, there was a decline in triglycerides, reaching significance at week 24 ($P = 0.05$) and week 48 ($P = 0.02$). There was no significant change in HDL-C levels with a small (10%) non-significant decline in LDL-C during the treatment period. There were no significant changes in other biochemical variables during the study period.

A small rise in haemoglobin concentrations was evident at week 48 ($P = 0.003$), which remained elevated during the recovery period. Haematocrit remained unchanged.

Body composition

There were no significant changes in body weight during the treatment or recovery periods. Likewise, body composition analysis showed no changes in fat free mass or percentage body fat (Table III).

Table III. Body composition data (weight, fat-free mass and % body fat) pre-treatment, 12 weekly during treatment and after 16 weeks of follow up

	Pre-treatment	12 weeks	24 weeks	36 weeks	48 weeks	Recovery
Weight (kg)	82.4 \pm 3.4	81.6 \pm 3.5	81.3 \pm 3.2	82.4 \pm 2.8	83.7 \pm 2.9	80.9 \pm 3.5
Fat-free mass (kg)	64.6 \pm 2.2	62.3 \pm 2.4	64.4 \pm 2.6	64.4 \pm 2.6	63.7 \pm 1.9	63.6 \pm 2.4
% Body fat	20.7 \pm 2.3	21.5 \pm 2.6	22.1 \pm 1.9	21.6 \pm 2.8	23.3 \pm 2.9	19.4 \pm 2.5

Data are presented as the mean \pm SEM. No significant changes indicated.

Table IV. Sexual behaviour pre-treatment, 12 weekly during etonogestrel/testosterone treatment and after 16 weeks of follow-up

	Pre-treatment	12 weeks	24 weeks	36 weeks	48 weeks	Recovery
Sexual behaviour	6.2 \pm 1.2	7.4 \pm 1.7*	6.5 \pm 1.8	5.3 \pm 0.8	5.3 \pm 1.5	4.8 \pm 0.9

Data are presented as the mean \pm SEM.

Sexual activity was assessed as the sum of acts of masturbation and intercourse during the preceding 2 weeks.

*A significant increase at week 12 of treatment ($P = 0.04$).

Sexual behaviour

There was a slight increase in sexual activity (recorded as the sum of number of acts of sexual intercourse and masturbation over the preceding 2 weeks) at week 12 of treatment ($P = 0.04$). No changes in sexual activity at other time points were observed during the study (Table IV).

Discussion

One of the major hurdles in the development of a hormonal male contraceptive is the need for sufficient and universal suppression of spermatogenesis. Caucasian populations have shown heterogeneous responses to both testosterone alone and testosterone in combination with progestogens (World Health Organization Task Force on Methods for the Regulation of Male Fertility, 1990; Anderson and Baird, 2002), although the addition of a progestogen has generally increased the proportion of men achieving azoospermia. We and others previously have demonstrated very high rates of azoospermia using oral desogestrel as the progestogen (Wu *et al.*, 1999; Anawalt *et al.*, 2000; Kinniburgh *et al.*, 2002). Administration of an implant preparation of etonogestrel, the active metabolite of desogestrel, also resulted in effective suppression of spermatogenesis (Anderson *et al.*, 2002). In the present study, we have explored further both the dose-response relationship and the duration of action of etonogestrel implants when administered with a depot testosterone preparation.

The present data demonstrate profound suppression of spermatogenesis with the combination of three etonogestrel implants and depot testosterone pellets, with all subjects achieving azoospermia. This compares favourably with our previous data using one (64% azoospermia) and two implants (75% azoospermia) over a 24 week period (Anderson *et al.*, 2002) and is similar to that achieved with an oral dose of 300 μ g desogestrel with the same regimen of testosterone administration (Kinniburgh *et al.*, 2002). Although sample sizes do not allow demonstration of statistically greater spermatogenic suppression with three than two etonogestrel implants, increased efficacy is supported by the more

consistent suppression of gonadotrophins and of both serum and seminal inhibin B with three implants. The onset of suppression was rapid, with all subjects having sperm concentrations of $<1 \times 10^6/\text{ml}$ by week 16 of treatment. However, the time taken to reach azoospermia was considerably more variable, with three men maintaining very low but detectable numbers of sperm in the ejaculate to up to 28 weeks. Similar data are evident from the recent Australian efficacy study (Turner *et al.*, 2003) despite the very rapid suppression achieved by that combination of testosterone pellets and depot medroxyprogesterone acetate (DMPA), whereby 94% of men achieved a sperm concentration of $<1 \times 10^6/\text{ml}$ within 3 months. This may have significant implications for the practicality of the method, depending on the threshold required for acceptable contraceptive efficacy (Nieschlag, 2002).

Serum etonogestrel concentrations of ~ 1200 and $500\text{--}800\text{ pg/ml}$ were reported for 300 and $150\text{ }\mu\text{g}$ oral desogestrel, respectively (Wu *et al.*, 1999; Anawalt *et al.*, 2000). In the present study, the serum etonogestrel concentration at 12 weeks was $\sim 600\text{ pg/ml}$. Thus the suppressive effect of this preparation is similar to that of $300\text{ }\mu\text{g}$ desogestrel per day, whereas the dose is similar to $150\text{ }\mu\text{g/day}$. Dose-sparing is also evident with this preparation of testosterone (Handelsman *et al.*, 1992), which maintains relatively stable serum concentrations and particularly avoids the supraphysiological peaks observed with esters such as testosterone enanthate (World Health Organization Task Force on Methods for the Regulation of Male Fertility, 1990). The dose of testosterone administered here has no significant suppressive effect on spermatogenesis when given alone (Handelsman *et al.*, 2000), and in combination with a progestogen may be the minimum effective dose. The advantageous features of this testosterone preparation will contribute to minimizing the intratesticular testosterone concentration which is recognized to be of importance in maximizing spermatogenic suppression (Meriggiola *et al.*, 2002; Zhang *et al.*, 2003).

The diurnal variation of testosterone concentrations in adult men has been well characterized (Faiman and Winter, 1971; Bremner *et al.*, 1983) if not understood. The dose of testosterone which is physiological is usually considered to be that which reproduces the peak concentration observed in men during the morning (Nieschlag *et al.*, 1992). This may result in the administration of a higher dose than that required for physiological replacement. In this study, we carried out a preliminary investigation of diurnal variation in serum testosterone before and during testosterone/progestogen administration, which we hypothesized would not be detectable during exogenous steroid administration if it was primarily due to variation in testosterone production rather than metabolism (Southren *et al.*, 1967). The data confirmed that the diurnal variation of testosterone was lost during treatment, at both 4 and 12 weeks. Testosterone concentrations at 4 weeks were similar to pre-treatment evening samples; however, they are probably lower than average over the duration of treatment. While the regimen used here provides the standard replacement dose for hypogonadal men (800 mg every 6 months; Behre *et al.*, 2004), administration of half the total

dose every 12 weeks will result in slight under-replacement over the initial 12 weeks, with steady state reached after the second administration. The average testosterone concentration following second administration was 15.5 nmol/l , which matches accurately the average 24 h concentration determined by frequent sampling in a group of young healthy men (Plymate *et al.*, 1989). This regimen may therefore closely replace testosterone production based on physiological diurnal production rather than morning peaks. The lack of changes in non-reproductive functions such as lipoproteins, haematocrit and body composition observed in this study is strong evidence that the dose administered here ($\sim 5\text{ mg/day}$ at steady state) provides close to physiological replacement, but this will need confirmation in longer studies assessing a wide range of androgen-dependent functions.

Gonadotrophin secretion was profoundly suppressed during treatment. This was particularly marked with LH. Suppression of FSH was more variable, but greater than with one or two implants (Anderson *et al.*, 2002). The 12 week testosterone administration regimen also appears more effective at preventing FSH escape than the same total dose administered at 24 week intervals (Turner *et al.*, 2003). Desogestrel and other progestogens may result in greater spermatogenic suppression than achieved by comparable gonadotrophin suppression using testosterone alone (McLachlan *et al.*, 2002), consistent with direct testicular effects on steroidogenesis (Satyaswaroop and Gurpide, 1978; El-Hefwany and Huhtaniemi, 1998; El-Hefwany *et al.*, 2000) or androgen metabolism (Mauvais-Jarvis *et al.*, 1974). In the present study, FSH was incompletely suppressed during weeks 24–48 in three subjects, only one of whom showed spermatogenic recovery. While adequate suppression of FSH is clearly necessary for achievement of azoospermia (Narula *et al.*, 2001; Weinbauer *et al.*, 2001), it appears that there is no clear threshold below which azoospermia can be confidently predicted, and that FSH suppression is only one of a number of potential determining factors for incomplete suppression or escape of spermatogenesis. Consistent with the reproducible suppression of LH, urinary excretion of epitestosterone fell to $\sim 10\%$ of pre-treatment values and remained at that level for the duration of treatment. Epitestosterone ($17\alpha\text{-hydroxyandrost-4-en-3-one}$) is a natural epimer of testosterone secreted predominantly by the testis (Kicman *et al.*, 1999) which therefore provides a measure of endogenous testicular secretion. Epitestosterone excretion during the present treatment regimen was similar to that previously reported during oral desogestrel/testosterone treatment of normal men (Kinniburgh *et al.*, 2002), and is significantly higher than in hypogonadal men (Kicman *et al.*, 1999). Direct measurement of intratesticular testosterone also indicates low ongoing testosterone production despite near complete LH suppression (McLachlan *et al.*, 2002).

The concentration of inhibin B provides an overall measure of Sertoli cell number and function including spermatogenesis (Anderson and Sharpe, 2000). While it would be expected that effective hormonal contraceptive regimens would result in significant falls in inhibin B concentrations, this has not always proved to be the case (Anawalt *et al.*,

1996; Anderson *et al.*, 1997; Zhengwei *et al.*, 1998; Büchter *et al.*, 1999; Martin *et al.*, 2000b; Kinniburgh *et al.*, 2002). We have not found significant changes in our previous studies with both oral desogestrel and etonogestrel implants despite the high prevalence of azoospermia. It is likely that changes in circulating inhibin B require profound regression of spermatogenesis more consistently throughout the testis than is achieved with some regimens (Anderson and Sharpe, 2000). This is supported by testis biopsy data showing variable degrees of spermatogenic regression between nearby seminiferous tubules despite induction of azoospermia (Zhengwei *et al.*, 1998; McLachlan *et al.*, 2002). It is possible that the fall in serum inhibin B contributed to the less consistent suppression of FSH than LH during the latter months of this study. The fall in inhibin B observed in the present study may reflect a greater consistency of suppression than is reflected purely by the prevalence of azoospermia. This is supported by the striking fall in the concentration of inhibin B in the ejaculate. A more variable fall was found in our previous study with one or two etonogestrel implants (Anderson *et al.*, 2002). In the present study, we confirm and enlarge on this finding that changes in seminal inhibin B are a sensitive window into the seminiferous epithelium, as seminal inhibin B was profoundly suppressed in all men to a median of <10 pg/ml at 24 weeks treatment. This is supported by observations in the individual who demonstrated recovery of spermatogenesis during treatment, as the appearance of sperm in the ejaculate intriguingly was preceded by a partial recovery of seminal plasma inhibin B. Interestingly, both serum and seminal inhibin B showed only limited recovery over 16 weeks, while sperm concentrations had largely returned to normal, indicating complex relationships between these various markers of testicular function. Further investigation is required to establish the time scale for recovery of the endocrine function of the seminiferous epithelium following gonadotrophin suppression.

Other approaches using long-acting preparations have involved implants and depot injections. Levonorgestrel has also been administered in implant formulation (Norplant II®), with azoospermia achieved in 35% of subjects when given with transdermal testosterone patches and 93% of subjects in combination with weekly testosterone enanthate (Gao *et al.*, 1999; Gaw Gonzalo *et al.*, 2002). The combination with testosterone implants or long-acting injectable preparations has yet to be investigated. 7 α -Methyl-19-nortestosterone (MENT), a synthetic androgen more potent than testosterone and resistant to 5 α -reduction (Sundaram *et al.*, 1993), has also been developed recently as an implant and a potential long-acting male contraceptive. However, even when up to four implants were used (a dose which resulted in significant effects related to excess androgenicity), 30% of men still had significant numbers of sperm in the ejaculate (von Eckardstein *et al.*, 2003) consistent with the limitations of an androgen-only approach in Caucasian men. Thus, of implant approaches to date, the combination presented in this study exhibits higher levels of spermatogenic suppression with a more favourable side effect profile than any of the others. This beneficial therapeutic ratio is likely to reflect the

pharmacokinetics of both the testosterone and progestogen preparations. Other promising long-term approaches include long-acting injectable testosterone undecanoate alone, achieving high levels of oligozoospermia and azoospermia among Chinese men (Gu *et al.*, 2003), the depot injectable combination of norethisterone enanthate and testosterone undecanoate (Kamischke *et al.* 2002), and DMPA with testosterone pellets (Turner *et al.*, 2003).

In conclusion, the results in this study demonstrate that administration of etonogestrel implants at an appropriate dose together with a long-acting testosterone preparation induces profound and consistent suppression of spermatogenesis that can be maintained for a period of 1 year. Whether this time period could be extended remains to be investigated. The maintenance of testosterone concentrations within the eugonadal range and the dose-sparing effects of the delivery methods involving constant release may contribute to the lack of non-reproductive effects. This approach may be a template as the basis for an acceptable, long-acting, and reversible male hormonal contraceptive.

Acknowledgements

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A multicentre study investigating subcutaneous etonogestrel implants with injectable testosterone decanoate as a potential long-acting male contraceptive

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BACKGROUND: The combination of etonogestrel implants with injectable testosterone decanoate was investigated as a potential male contraceptive. **METHODS:** One hundred and thirty subjects were randomly assigned to three treatment groups, all receiving two etonogestrel rods (204 mg etonogestrel) and 400 mg testosterone decanoate either every 4 weeks (group I, $n = 42$), or every 6 weeks (group II, $n = 51$) or 600 mg testosterone decanoate every 6 weeks (group III, $n = 37$) for a treatment period of 48 weeks. **RESULTS:** One hundred and ten men completed 48 weeks of treatment. Sperm concentrations of $<1 \times 10^6/\text{ml}$ were achieved in 90% (group I), 82% (group II) and 89% (group III) of subjects by week 24. Suppression was slower in group II, which also demonstrated more frequent escape from gonadotrophin suppression than groups I and III. Peak testosterone concentrations remained in the normal range throughout in all groups. Mean trough testosterone concentrations were initially subphysiological but increased into the normal range during treatment. Mean haemoglobin levels increased in group I, and a non-significant increase in weight and decline in high-density lipoprotein cholesterol was observed in all groups. Fourteen subjects discontinued treatment due to adverse events. **CONCLUSIONS:** Subcutaneous etonogestrel implants in combination with injectable testosterone decanoate resulted in profound suppression of spermatogenesis that could be maintained for up to 1 year. Efficacy of suppression was less in group II, probably due to inadequate testosterone dosage. This combination has potential as a long-acting male hormonal contraceptive.

Key words: etonogestrel/gestogen/male contraceptive/spermatogenesis/testosterone decanoate

Introduction

The concept of hormonal male contraception is based upon the administration of exogenous steroid to suppress pituitary gonadotrophins, with the subsequent suppression of spermatogenesis (Anderson and Baird, 2002; Nieschlag *et al.*, 2003; Kamischke and Nieschlag, 2004; Wang and Swerdloff, 2004). Earlier approaches involved the administration of androgen alone (World Health Organization Task Force on Methods for the Regulation of Male Fertility, 1990, 1996). However, low rates of spermatogenesis were maintained in approximately one third of Caucasians, with the resulting risk of pregnancy. Furthermore, supraphysiological androgen levels resulted in significant side-effects on skin, haematopoiesis and serum lipoproteins (Wu *et al.*, 1996). The administration of a second agent, such as a progestogen, improves the degree of spermatogenic suppression and

permits lowering of the dose of testosterone to nearer physiological replacement (Bebb *et al.*, 1996; Handelsman *et al.*, 1996; Meriggiola *et al.*, 1996, 2002). Several progestogens have been investigated, including levonorgestrel (Bebb *et al.*, 1996), cyproterone acetate (Meriggiola *et al.*, 1996, 1998), medroxyprogesterone acetate (Knuth *et al.*, 1989; World Health Organization Task Force on Methods for the Regulation of Male Fertility, 1993; Handelsman *et al.*, 1996; Turner *et al.*, 2003), norethisterone (Kamischke *et al.*, 2001, 2002) and desogestrel (Wu *et al.*, 1999; Anawalt *et al.*, 2000; Kinniburgh *et al.*, 2002). Results with desogestrel have been promising, with near universal suppression of spermatogenesis.

Etonogestrel, the active metabolite of desogestrel, is now licensed as a long-acting implant preparation for use as a female contraceptive (Implanon®). In addition to convenience and

optimum levels of compliance, administration as an implant may confer advantages over an oral preparation, allowing dose-sparing and avoiding liver exposure by bypassing first-pass metabolism, thus minimising adverse metabolic effects. This has indeed been demonstrated in a recent study using two 68 mg etonogestrel implants with depot testosterone (Anderson *et al.*, 2002a). Despite using lower exposure to etonogestrel in comparison with oral desogestrel, similar efficacy in suppression of spermatogenesis was observed with reduced non-reproductive effects (Kinniburgh *et al.*, 2002). In that study, suppression of spermatogenesis was greater with two implants than with one (75 versus 64% azoospermia). We therefore hypothesized that suppression of spermatogenesis may be improved by further increasing this dose by 50%, and two larger implants (each containing 102 mg etonogestrel) were administered in the present study.

The lack of availability of a convenient long-acting injectable testosterone preparation has been a major obstacle to the development of hormonal male contraception. Previous studies with testosterone enanthate relied on weekly injection intervals. Not only may this be unacceptable to volunteers, but supraphysiological testosterone peaks were observed as well. Testosterone decanoate (TD) is one of the testosterone esters contained in Sustanon®, which has been used for several years in the treatment of hypogonadism. Preliminary data demonstrated that 400 mg administered every 4 weeks with etonogestrel implants resulted in good spermatogenic suppression (Anderson *et al.*, 2002b). We therefore further explored the optimal dose of this androgen in three administration regimens: 400 mg every 4 weeks (group I), 400 mg every 6 weeks (group II) and 600 mg every 6 weeks (group III).

This combination of TD with etonogestrel implants was investigated in a Phase IIb multicentre trial. The primary objective was to assess its effects on the suppression of spermatogenesis in the three treatment groups. Secondary objectives included evaluation of the suppression of gonadotrophins, the pharmacokinetics of the TD regimes, and safety monitoring.

Subject and methods

Subjects

One hundred and thirty subjects were recruited from six centres in Europe and the USA. The inclusion criteria included age ≥ 18 and ≤ 45 years; mentally and physically healthy; BMI ≥ 18 and ≤ 32 kg/m²; normal semen analysis on two occasions (examination within 60 min, based on WHO criteria (World Health Organization, 1999) for sperm concentration and WHO criteria or local reference ranges for sperm motility and morphology); normal hormone (FSH, LH and testosterone) concentrations based on local reference ranges; and willingness to provide written informed consent. Men in a sexual relationship at study inclusion had to be willing to use a reliable form of contraception. Each subject gave informed written consent. Ethical approval was received from each centre's local Ethical Review Committee.

Medication

Etonogestrel implants were 6 cm long and each contained 102 mg etonogestrel (Organon, Oss, The Netherlands). They were inserted under local anaesthetic under the skin of the medial aspect of the non-dominant upper arm and removed following the 48-week treatment

period. Testosterone decanoate (3-oxo-androst-4-en-17 β -yl decanoate, TD) at a concentration of 200 mg/ml was administered by deep intramuscular injection on the day of etonogestrel implant insertion. Subjects re-attended every 4 or 6 weeks (± 3 days) thereafter, depending on the treatment group, for subsequent injections.

Study design

The study was an open-label randomized multicentre trial investigating the suppressive effects of etonogestrel subcutaneous implants with injectable TD on spermatogenesis. The study also aimed to investigate the suppressive effect of these regimes on gonadotrophins as well as the safety and pharmacokinetics of this regime. Subjects were randomized into three treatment groups. All groups received two etonogestrel implants. Group I received 400 mg TD every 4 weeks, group II 400 mg TD every 6 weeks and group III 600 mg TD every 6 weeks.

Subjects were reviewed every 4 weeks in the first 24 weeks of the treatment phase and during recovery. During weeks 24–48, subjects attended every 4 or 6 weeks depending on the treatment group. At each visit, subjects submitted a semen sample and safety assessments were performed, checking routine laboratory parameters, including prostate-specific antigen, inspection of the implant site, and recording of adverse events and any concomitant medications. Physical examination was performed every 12 weeks (with andrological examination assessing testes, and prostate assessment by digital examination or transrectal ultrasonography at weeks 24, 48 and final assessment). During the treatment phase, venepuncture was performed for hormone measurements at weeks 1, 2, 4, 8, 12, 13, 14, 16, 20, 24, 36 and 48 in the 4-week group and at weeks 1, 2, 4, 6, 12, 13, 14, 16, 18, 24, 36 and 48 in the 6-week group. At most of these time points, trough levels were measured because blood sampling occurred prior to the TD injection. Peak testosterone levels were measured at weeks 1 and 2 (after the first injection) and at weeks 13 and 14 after the third (4 weeks group) and second (6 weeks group) injections. Etonogestrel concentrations were assessed at weeks 1, 2, 4, 8, 12, 24, 36 and 48. During the follow-up phase, subjects attended every 4 weeks until week 16, when they underwent final assessment if sperm concentration was greater than 20×10^6 /ml or they continued until week 24. Any subject not recovered at week 24 entered an extended phase of follow-up of indefinite duration.

Semen analysis

Semen samples were submitted after 2–7 days of abstinence and assessed for semen volume, sperm concentration, morphology and motility by WHO criteria. Motility was assessed within 60 min of ejaculation. Azoospermia was confirmed by centrifugation of the entire ejaculate and thorough and comprehensive examination of the pellet. Motility and morphology were not assessed during treatment because, as a result of profound suppression, there were insufficient sperm to enable accurate assessment.

Assays

Blood samples were separated by centrifugation and serum was stored at -20°C prior to shipping to a central laboratory (Organon) for assay. Serum gonadotrophins and sex hormone-binding globulin (SHBG) were determined by highly sensitive immunofluorometric assays (Delfia; Perkin Elmer). For FSH and LH respectively, assay sensitivities were 0.25 IU/l and 0.52 IU/l and intra- and inter-assay coefficients of variation (CVs) 1.9–7.6% and 2.6–4.9%. The lower limit of quantification for SHBG was 6.25 nmol/l, with intra- and inter-assay CVs of 3.2–5.0%. Testosterone was determined by capillary gas chromatography–mass spectrophotometry with an assay sensitivity of 0.35 nmol/l

and intra- and inter-assay CVs of 4.5–21.9%. Etonogestrel was measured by in-house radioimmunoassay (Organon) with a lower limit of quantification of 30.0 pg/ml. Samples were analysed locally for routine haematological and biochemical values, including prostate-specific antigen, cholesterol, high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol at 12 weekly intervals.

Behavioural assessment

Sexual function and mood were investigated before treatment, at weeks 12, 24, 36 and 48 of treatment, week 4 of follow-up and at final assessment. Sexual function was assessed by means of the Derogatis Interview for Sexual Functioning—Self Report (DISF-SR) (Derogatis, 1997). Questions on mood were assessed with an unvalidated questionnaire, and included questions on irritability, depression, fatigue and aggression. Moreover, some questions on local tolerance of the injections were asked.

Statistical analysis

Statistical analysis was performed by Organon. The efficacy results of the intention-to-treat group are presented. Frequencies of subjects with suppression of sperm concentration to a specified level and a certain time point were compared by means of Fisher's exact test with Bonferroni correction. Survival analyses were performed and, because of departure from the proportional hazards assumption, log-rank tests were used for comparison. Mean values of sperm and hormone concentrations, biochemistry, haematology and physical parameters were analysed by repeated measures analysis of variance and paired *t*-tests using Tukey's multiple comparison procedure. *P*-values presented for hormones, biochemistry, haematology and physical parameters were not corrected for multiple testing. However, multiplicity was taken into account by regarding a result statistically significant if $P < 0.0001$ (which would correspond to Bonferroni corrected *P*-values below 0.05). Values were expressed as the arithmetical mean \pm SEM. Hormone concentrations below the detection level were allocated the value of half of the lower limit of detection.

Results

Subjects

Pretreatment values for the subjects in each group are shown in Table I. There were no significant differences in age, BMI, sperm density and LH, FSH and testosterone concentrations between the three treatment groups.

One hundred and thirty subjects were randomized to the three groups, as follows: group I ($n = 42$), II ($n = 46$) and III ($n = 42$). Five subjects in group III were treated erroneously with 400 mg TD instead of 600 mg TD and therefore were analysed in group II, resulting in 42, 51 and 37 subjects in the

respective groups. In total, 119 subjects completed 24 weeks of treatment and 110 subjects completed the treatment period (84.6%): 33 subjects in group I (78.6%), 43 subjects in group II (84.3%) and 34 subjects in group III (91.9%). Overall, compliance with study medication was good, with 100% compliance with TD injections in group II and almost 99% compliance in the other two treatment groups.

Sperm concentrations

All men demonstrated a profound suppression of spermatogenesis (Figure 1a, Table II). By week 24, azoospermia was achieved in 28 (71.8%), 25 (55.6%) and 24 (68.6%) subjects in groups I, II and III respectively (Figure 1b). The extents of suppression in groups I and III at week 24 were similar and appeared greater than the suppression demonstrated by group II, although there was no statistically significant difference between suppression at weeks 16 or 24. At week 24, sperm concentrations had fallen to $<1 \times 10^6$ /ml in 89.7% (group I), 82.2% (group II) and 88.6% (group III) of subjects (Figure 1c). Four subjects in group I, five in group II and three in group III maintained sperm concentrations of $>3 \times 10^6$ /ml at week 24; all but four of these proceeded to concentrations close to azoospermia by week 48. Sperm concentrations further decreased in all groups after week 24, with azoospermia achieved in 81% (group I), 78% (group II) and 85% (group III) of subjects by the end of the treatment period. Once subjects were azoospermic, this was maintained at all subsequent visits in 56% (20/36 subjects, group I), 72% (26/36 subjects, group II) and 80% (24/30 subjects, group III) of subjects. Using the threshold of $<1 \times 10^6$ /ml, faster suppression of spermatogenesis was observed in groups I and III, the median number of days to reach this threshold being 59 (group I), 84 (group II) and 61 (group III) respectively. However, suppression to azoospermia occurred in a similar time for all three groups, the median number of days to reach this being 114 (group I), 118 (group II) and 113 (group III).

Overall, 102 (81%) men recovered within 24 weeks of follow-up. At that time, 77% (group I), 82% (group II) and 83% (group III) of subjects had reached normal sperm concentration by WHO criteria ($>20 \times 10^6$ /ml) in at least one sample. All of the remaining subjects except three recovered spermatogenesis within 52 weeks. Two of them recovered within 69 weeks and one subject discontinued follow-up before a semen sample of 20×10^6 /ml was obtained because his partner was pregnant (last sperm concentration was 18.3×10^6 /ml at 69 weeks of follow-up). Recovery was faster in group II (400 mg/6 weeks) than in groups I and III (at week 24; $P = 0.01$), with a median time to recovery of approximately 130 days in all treatment groups (Figure 2).

Reproductive hormones

Profound suppression of both LH and FSH was observed in all three treatment groups (Figure 3a and b). Gonadotrophin concentrations were suppressed to the level of detection by week 4 in the majority of subjects and remained suppressed throughout the treatment period. Suppression was, however, less consistent in the 400 mg/6 weeks group (group II), with more frequent fluctuation or 'escape' being observed. Recovery of both LH

Table I. Pretreatment values of subjects in the three treatment groups

Pretreatment value	Group I ($n = 41$)	Group II ($n = 51$)	Group III ($n = 37$)
Age (years)	30.8 \pm 0.9	31 \pm 0.8	31.5 \pm 1.1
BMI (kg/m ²)	24.6 \pm 0.4	24.7 \pm 0.4	25.0 \pm 0.5
LH (IU/l)	3.6 \pm 0.3	3.6 \pm 0.2	3.7 \pm 0.3
FSH (IU/l)	3.4 \pm 0.3	3.2 \pm 0.2	3.6 \pm 0.3
Testosterone (nmol/l)	19.2 \pm 1.3	18.7 \pm 0.7	20.5 \pm 1.3
Sperm density ($\times 10^6$ /ml)	72.6 \pm 6.6	90.6 \pm 7.9	75.2 \pm 7.7

Data are mean \pm SEM.

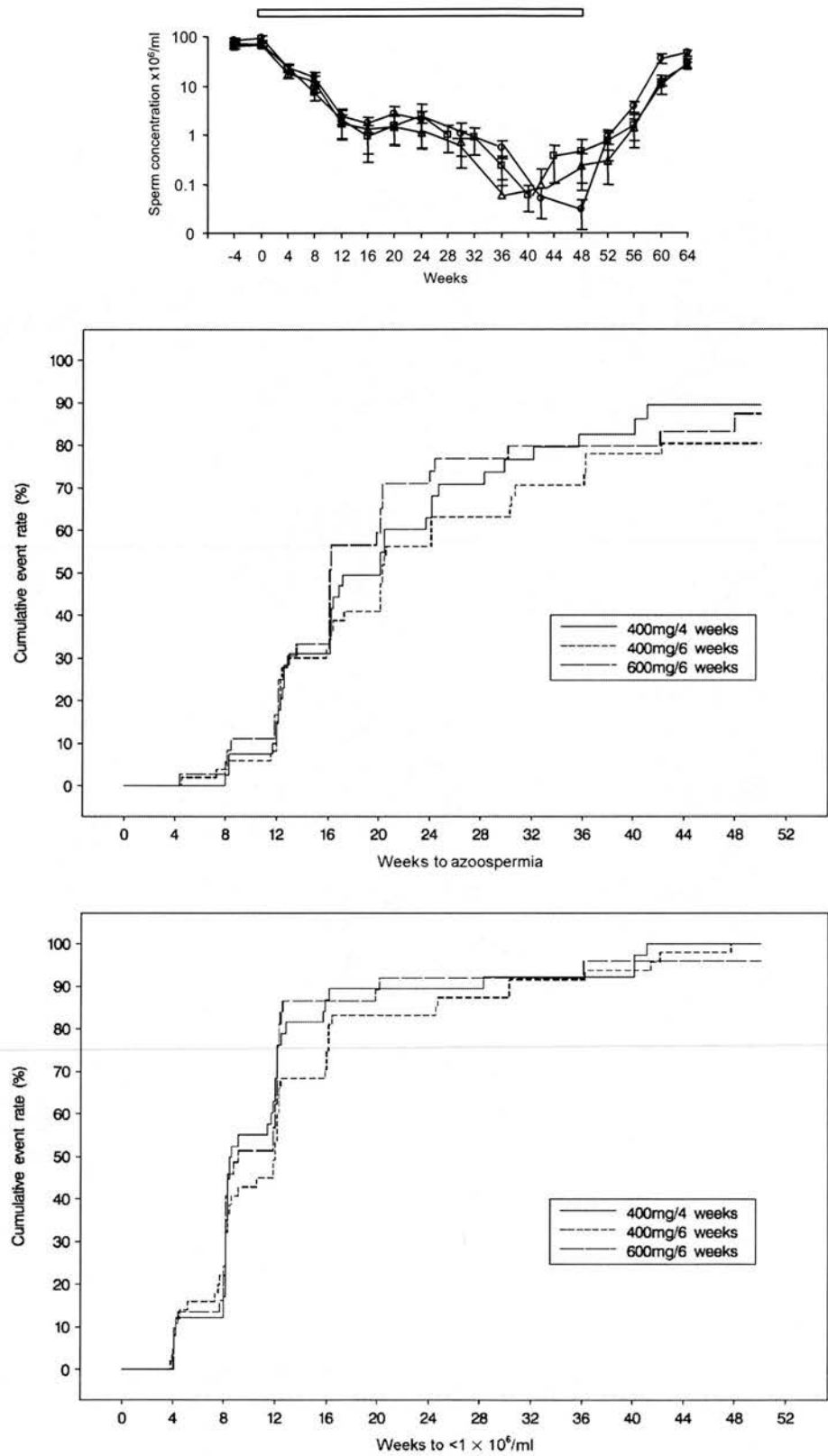
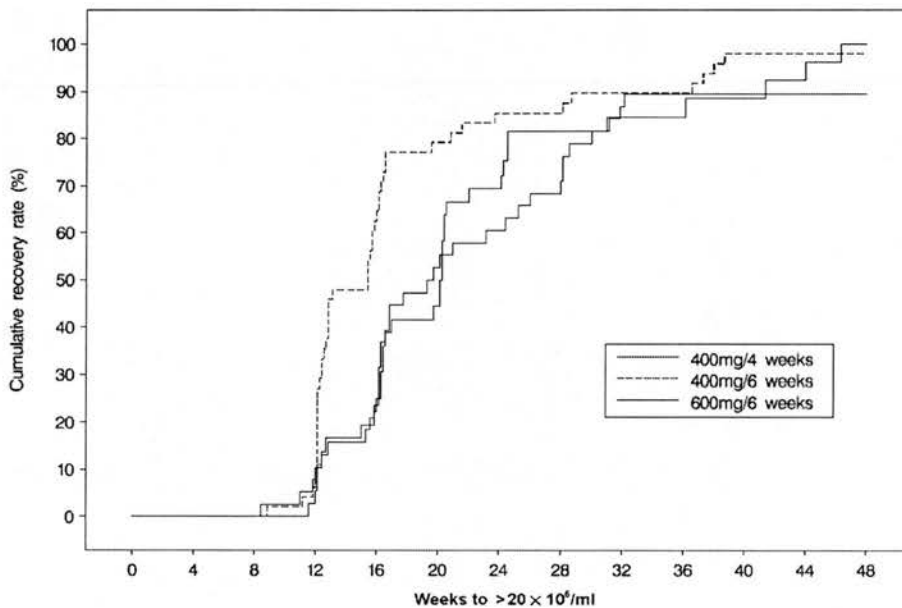


Figure 1. (a) Sperm concentrations during etonogestrel/TD treatment and recovery. Note the log scale on the ordinate. Data are mean \pm SEM. Group I, 400 mg TD/4 weeks; group II, 400 mg TD/6 weeks; group III, 600 mg TD/6 weeks. (b) Achievement of azoospermia and (c) sperm concentration of $<1 \times 10^6$ /ml during treatment. Panels b and c show cumulative event rates assessed during treatment by Kaplan–Meier estimation. Treatment groups are indicated in the legends.

Table II. Percentage of subjects suppressed to sperm concentration targets, receiving treatment with etonogestrel implants and i.m. TD at the doses indicated (intention-to-treat group)

Treatment group	Week	Percentage of subjects reaching the suppression targets				
		<i>n</i>	Azoospermia	$<0.1 \times 10^6/\text{ml}$	$<1 \times 10^6/\text{ml}$	$<3 \times 10^6/\text{ml}$
400 mg/4 weeks	8	39	10.3	43.6	56.4	69.2
	16	37	48.6	81.1	89.2	91.9
	24	39	71.8	87.2	89.7	89.7
	48	31	80.6	93.5	93.5	93.5
400 mg/6 weeks	8	49	4.1	22.4	40.8	51.0
	16	44	38.6	61.4	81.8	84.1
	24	45	55.6	71.1	82.2	88.9
	48	40	77.5	92.5	100	100
600 mg/6 weeks	8	36	11.1	38.9	50.0	63.9
	16	34	52.9	85.3	88.2	94.1
	24	35	68.6	80.0	88.6	91.4
	48	33	84.8	90.9	93.9	93.9

**Figure 2.** Achievement of recovery of spermatogenesis to concentrations of $>20 \times 10^6/\text{ml}$. This is a cumulative event rate assessed during treatment by Kaplan–Meier estimation. Treatment groups are indicated in the legends.

and FSH was faster in group II than in groups I and III ($P < 0.05$ at 4 weeks of follow-up), with no significant difference in recovery between groups I and III. There were no statistically significant differences between groups at the end of follow-up or when comparing final visit with pretreatment gonadotrophin concentrations.

Testosterone

Fluctuations in testosterone concentrations were observed, in keeping with the scheduling of TD injections (Figure 4). Mean peak testosterone concentrations remained within the physiological range (9.85–35.57 nmol/l) for all groups throughout the treatment period. Mean trough testosterone concentrations gradually increased over the time-course of the study, and were initially subphysiological in all three treatment groups (Figure 4). In group II, mean trough testosterone concentrations remained

below the physiological range until between week 24 and 36, whereas in groups I and III mean trough testosterone concentrations were in the normal range between week 12 and 16.

Other hormones

Peak mean etonogestrel concentrations were measured 1 week after insertion of the implants and demonstrated similar concentrations in all three treatment groups, of approximately 800–900 pg/ml. Thereafter, there was a gradual decline to approximately 300 pg/ml after 48 weeks of treatment.

Biochemistry and haematology

Serum SHBG concentrations decreased by approximately 30% within 8 weeks of treatment in all three treatment groups, remaining so until the end of treatment and returning to baseline

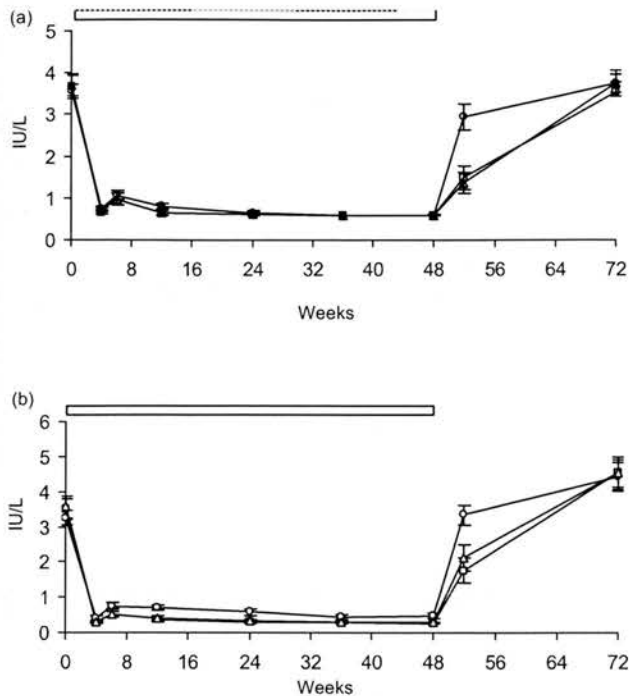


Figure 3. (a) Serum LH and (b) FSH concentrations during etonogestrel/TD treatment and recovery. Data are mean \pm SEM. Treatment period is indicated by a double bar. Group I, 400 mg/4 weeks; group II, 400 mg/6 weeks; group III, 600 mg/6 weeks.

concentrations thereafter. There was a non-significant decline in total cholesterol (approximately 4%) and HDL cholesterol (approximately 15%) in all groups returning to baseline levels following treatment (Table III). Mean haemoglobin concentrations increased in all groups (group I, 5%; group II, 2%; group III, 4%) during treatment, reaching statistical significance when compared with baseline in group I at 48 weeks ($P < 0.0001$), and returning to baseline levels at the final follow-up visit. A small increase in mean haematocrit was also observed in groups I (4%) and III (1%) during treatment; values returned to baseline

thereafter, although this was statistically not significant. No relevant changes in other parameters were observed (Table III).

Physical examination and behaviour

Testicular volume decreased by approximately 25% in all groups during treatment, returning to pretreatment volumes during the recovery phase (Table IV). There was no significant change in prostate volume, prostate-specific antigen or blood pressure throughout the study period. There was a slight increase in weight (5% in group I, 3.5% in group II and 5% in group III) that did not reach statistical significance. There was no significant change in overall mood scores across treatment groups throughout the study. There were no changes in the overall scores from the Derogatis Interview for Sexual Function throughout treatment or follow-up. Similarly, there were no differences in mean subscores for different DISF-SR functions.

Discontinuations and side-effects

Overall, 20 subjects discontinued the study in the respective groups as follows: nine in group I, eight in group II and three in group III. The most frequent reason for discontinuation was an adverse event (14 subjects, 10.8%), other reasons being non-compliance with the protocol or moving to another area. In group III, only one subject discontinued due to an adverse event, which was impotence. One other subject discontinued in group II due to impotence. Other adverse events that led to discontinuation were: aggressive reaction (with or without nervousness or emotional lability) (3); implant complication (2); depression (2); emotional lability (2); arthralgia (1); laryngitis (1); and myocarditis (1).

Four of the seven subjects with emotional events discontinued prior to week 24 of treatment.

Among the possible side-effects that did not lead to discontinuation, the most frequently reported were acne and increased sweating, mood changes, weight increase, and mild reactions related to implant insertion or removal. Most of the latter events concerned mild itching, pain, swelling or excess fibrous tissue, which complicated removal.

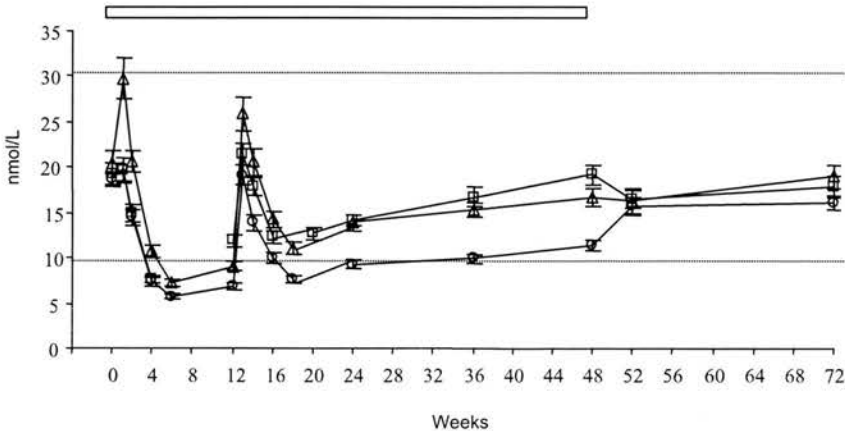


Figure 4. Serum testosterone concentrations (nmol/L) during etonogestrel/TD treatment and recovery. Data are mean \pm SEM. Treatment period is indicated by double bars and normal ranges by broken lines. Group I, 400 mg/4 weeks; group II, 400 mg/6 weeks; group III (600 mg/6 weeks).

Table III. Haematological and biochemical parameters during treatment and final visit of the follow-up phase

Phase	Group I (400 mg/4 weeks)				Group II (400 mg/6 weeks)				Group III (600 mg/6 weeks)			
	Treatment		Recovery		Treatment		Recovery		Treatment		Recovery	
	0	24	48	Final	0	24	48	Final	0	24	48	Final
Haemoglobin (g/l)	151.5 ± 1.1	156.1 ± 1.3	158.7 ± 1.4 ^a	154.6 ± 1.2	151.0 ± 1.3	152.1 ± 1.3	154.5 ± 1.2	152.8 ± 1.3	150.4 ± 1.4	154.2 ± 1.4	156.9 ± 1.5	151.3 ± 1.3
Haeamotocrit	0.45 ± 0.00	0.46 ± 0.00	0.47 ± 0.00	0.45 ± 0.00	0.45 ± 0.00	0.45 ± 0.00	0.45 ± 0.00	0.45 ± 0.00	0.44 ± 0.00	0.45 ± 0.00	0.46 ± 0.00	0.44 ± 0.00
Cholesterol (mmol/l)	4.7 ± 0.1	4.4 ± 0.2	4.5 ± 0.2	4.8 ± 0.2	4.6 ± 0.1	4.6 ± 0.1	4.5 ± 0.1	4.8 ± 0.1	4.7 ± 0.1	4.6 ± 0.2	4.5 ± 0.2	4.8 ± 0.1
HDL cholesterol (mmol/l)	1.4 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.4 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	1.2 ± 0.0	1.3 ± 0.1
LDL cholesterol (mmol/l)	2.8 ± 0.1	2.7 ± 0.1	2.9 ± 0.1	2.9 ± 0.1	2.7 ± 0.1	2.8 ± 0.1	2.8 ± 0.1	2.9 ± 0.1	2.7 ± 0.1	2.8 ± 0.1	2.8 ± 0.1	2.9 ± 0.1
Triglycerides	1.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
SHBG (nmol/l)	32.6 ± 2.0	24.9 ± 2.3 ^a	25.4 ± 2.7 ^a	31.2 ± 2.1	37.1 ± 2.1	27.1 ± 1.6 ^a	27.4 ± 1.8 ^a	33.3 ± 2.0	38.6 ± 2.6	27.7 ± 2.0 ^a	26.4 ± 2.3 ^a	37.7 ± 2.8
ALT (U/l)	25.1 ± 1.9	25.4 ± 2.0	26.0 ± 2.5	29.3 ± 2.2	23.0 ± 1.8	24.5 ± 2.1	24.2 ± 2.4	29.4 ± 2.1	23.2 ± 1.6	24.9 ± 1.9	24.9 ± 2.0	31.4 ± 3.2
AST (U/l)	22.0 ± 1.4	22.8 ± 2.4	20.8 ± 1.3	23.5 ± 1.3	23.4 ± 1.4	21.8 ± 1.3	21.4 ± 1.2	24.4 ± 1.3	22.6 ± 1.3	21.2 ± 1.3	21.1 ± 1.3	26.3 ± 1.8
GGT (U/l)	21.7 ± 1.6	27.1 ± 2.9	27.8 ± 2.9	27.5 ± 2.8	23.7 ± 2.1	29.2 ± 3.3	28.1 ± 2.7	28.4 ± 3.1	20.5 ± 1.6	26.2 ± 2.1	28.9 ± 2.8	27.4 ± 3.0
Bilirubin (μmol/l)	15.0 ± 1.0	14.9 ± 1.2	17.3 ± 1.3	13.8 ± 1.0	14.5 ± 0.9	13.9 ± 0.8	15.9 ± 1.1	13.8 ± 0.9	15.9 ± 1.3	15.5 ± 1.4	15.8 ± 1.3	15.2 ± 1.4

Data are mean ± SEM for AST group. ^aStatistically significant ($P < 0.0001$) compared with baseline. There were no statistically significant differences between the three groups at any treatment point.

GGT = γ-glutamyl transpeptidase.

Table IV. Clinical parameters at baseline during treatment and final visits in the follow-up phase

Phase	Group I (400 mg/4 weeks)				Group II (400 mg/6 weeks)				Group III (600 mg/6 weeks)			
	Treatment		Recovery		Treatment		Recovery		Treatment		Recovery	
	0	24	48	Final	0	24	48	Final	0	24	48	Final
Weight (kg)	79.0 ± 1.6	81.7 ± 1.5	82.9 ± 1.7	80.0 ± 1.4	79.4 ± 1.7	81.9 ± 1.8	82.0 ± 1.9	81.5 ± 1.6	79.3 ± 1.7	83.3 ± 1.7	83.2 ± 1.9	81.4 ± 1.9
Prostate volume (ml)	18.6 ± 1.0	19.6 ± 1.0	19.6 ± 1.3	18.2 ± 1.0	17.7 ± 1.0	18.6 ± 1.2	17.9 ± 0.7	17.1 ± 0.9	19.2 ± 1.1	19.6 ± 1.0	18.6 ± 1.1	17.3 ± 0.8
prostate-specific antigen (ng/ml)	0.9 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.0	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
Mean testicular volume (ml)	22.6 ± 0.7	17.0 ± 0.8 ^a	16.4 ± 0.9 ^a	22.4 ± 0.9	24.9 ± 0.7	18.7 ± 0.8 ^a	17.8 ± 0.8 ^a	24.3 ± 0.8	24.8 ± 0.7	17.4 ± 0.9 ^a	16.2 ± 0.9 ^a	23.0 ± 0.8
Overall DISF score	103.2 ± 2.4	103.0 ± 3.0	102.3 ± 3.4	97.8 ± 2.7	101.7 ± 2.2	102.3 ± 2.8	100.8 ± 2.7	96.3 ± 3.1	103.5 ± 2.5	107.3 ± 3.0	107.9 ± 3.3	103.3 ± 3.2

Data are mean ± SEM for the AST group. ^aStatistically significant from baseline ($P < 0.0001$). DISF = Derogatis Interview for Sexual Functioning—Self-Report.

Discussion

The combination of etonogestrel implants and injectable TD resulted in profound suppression of spermatogenesis, comparable to other approaches using a combination of androgen and progestogen for male hormonal contraception (Anderson and Baird, 2002; Nieschlag *et al.*, 2003). In this study, comparatively large numbers of subjects were studied for a longer treatment period of 48 weeks, which has been investigated in only a few studies to date (Gu *et al.*, 2003; Turner *et al.*, 2003; Brady *et al.*, 2004). Overall, azoospermia was achieved by 24 weeks in 55–72% of subjects, increasing to 78–85% of subjects by week 48. In the only contraceptive efficacy study to date using an androgen–progestogen combination, no pregnancies were reported with 426 months of contraceptive exposure in 51 men using the suppression threshold of $1 \times 10^6/\text{ml}$ (Turner *et al.*, 2003). Using the combination of etonogestrel implants with TD, this degree of suppression was achieved in approximately 90% of subjects by week 24, demonstrating that it may have potential as an efficacious contraceptive method.

While all men demonstrated suppression of spermatogenesis, there were differences in the rate and degree of suppression between treatment groups. At week 24, suppression to azoospermia was lower in group II (56%) than in groups I (72%) and III (69%) although this difference was less by the end of the treatment period. There was also a lesser suppression of gonadotrophins in group II, with a greater fluctuation than in the other 2 groups. This may be attributed to the less frequent administration of testosterone in that group. Indeed, trough testosterone concentrations in group II remained subphysiological until weeks 24–36, and the improved spermatogenic suppression in the latter half of the study may have been related to the higher trough testosterone levels. Comparable differences in the degree of spermatogenic suppression in response to different TD dose regimens has recently been demonstrated using oral etonogestrel with TD (Hay *et al.*, 2005), although overall rates of suppression were slightly higher. This suggests that modifications to the dose of either the testosterone or progestogen component may improve the rate and degree of suppression.

Although the testosterone dosages used in the current study maintain peak testosterone concentrations within the physiological range, trough concentrations showed a continuing slight increase during the length of the treatment period. While it therefore appears that yet longer studies are required to fully investigate the pharmacokinetics of this preparation, the present data clearly indicate that TD has advantages over previously available testosterone preparations. Similarly, testosterone undecanoate has improved pharmacokinetics compared with testosterone enanthate (Chen *et al.*, 1991; Li *et al.*, 1994). In studies with a repeated injection schedule of testosterone undecanoate (Nieschlag *et al.*, 1999; Zhang *et al.*, 1999; Gu *et al.*, 2003, 2004), cumulative effects were also observed. Previous studies in which peak testosterone levels were not assessed (Zhang *et al.*, 1999) may have underestimated the total exposure to testosterone. The slow improvement of testosterone preparations has been a significant barrier to the development of hormonal male contraception, and it appears that

these newer preparations offer considerable advantages. The other long-acting testosterone preparation, subcutaneous pellets (Handelsman *et al.*, 1990), also has high efficacy in the context of male contraception, the zero-order release allowing dose-sparing (Handelsman *et al.*, 1992; McLachlan *et al.*, 2000). No studies have compared these preparations directly, but the considerable improvements in testosterone delivery exemplified by this TD preparation now make detailed investigation of the testosterone regimen of importance.

A proportion (21/130, 16%) of men in all three groups entered a period of prolonged follow-up with delayed recovery in spermatogenesis. This has also been observed in previous studies with gestogen–androgen combinations (Brenner *et al.*, 1977). Such prolonged recovery has not been observed in previous studies using a similar dose of desogestrel (Wu *et al.*, 1999; Anawalt *et al.*, 2000; Kinniburgh *et al.*, 2002). However, with the exception of the extension phase in the study by Kinniburgh *et al.*, these studies were of a duration of 24 weeks and not 48, and it cannot be ascertained that their follow-up was complete beyond the usual period of 16–24 weeks. Although the effects of progestogens on the hypothalamus and pituitary in men are unclear, there is evidence to support a possible direct effect of progestogens on the testis. A non-classical progesterone receptor has been identified in spermatozoa (El-Hefwany *et al.*, 2000) and in rat Leydig cells (Rossato *et al.*, 1999) and progesterone has been demonstrated to down-regulate LH receptor expression and function *in vitro* (El-Hefwany and Huhtaniemi, 1998). Desogestrel may also have a direct effect on Leydig cell steroidogenesis (Satyawarup and Gurpide, 1978), further decreasing intratesticular testosterone concentrations and having an inhibitory effect on spermatogenesis. However, as etonogestrel has a half-life of 23.8 hours (Bergink *et al.*, 1990), it is not likely that such mechanisms alone may account for the prolonged recovery of spermatogenesis. Recovery data in previous studies of testosterone esters alone are incomplete. In the WHO study using testosterone enanthate alone (World Health Organization Task Force on Methods for the Regulation of Male Fertility, 1996), although the mean time to recover to normal levels was 16 weeks, 20% of subjects were lost to follow-up. Similarly, more recently in a large efficacy study in China administering testosterone undecanoate alone, the recovery period was 12 months and no information was given on the follow-up of the subjects who discontinued from the study (Gu *et al.*, 2003). The mechanisms of heterogeneity in the response to hormonal regimes discussed above has been a subject of considerable debate (Handelsman *et al.*, 1995; Anderson *et al.*, 1996; Yu and Handelsman, 2001; von Eckardstein *et al.*, 2002) and it appears that this heterogeneity in response may also be true of recovery.

Similarly to studies using androgen–gestogen combinations (Bebb *et al.*, 1996; Wu *et al.*, 1999; Anawalt *et al.*, 2000), a decline in HDL cholesterol and small increase in body weight was observed in all groups. Although demonstrating comparatively greater selectivity for the progesterone receptor than other synthetic progestogens (Phillips *et al.*, 1990), etonogestrel has some affinity for the androgen receptor and may therefore contribute to these androgenic effects. In previous studies using this combination, weight gain and decline in

HDL cholesterol concentrations were dependent on the doses of both desogestrel and testosterone (Anawalt *et al.*, 2000).

In conclusion, this study demonstrates profound suppression of spermatogenesis with the combination of etonogestrel implants and testosterone decanoate. Efficacy in spermatogenic suppression was greater in groups I and III than in group II, which received 400 mg TD every 6 weeks, indicating that this is a submaximal regimen, and this was supported by pharmacokinetic analysis. This combination is a valuable approach and may lead to the development of a safe and effective long-term male hormonal contraceptive.

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A Multicenter Phase IIb Study of a Novel Combination of Intramuscular Androgen (Testosterone Decanoate) and Oral Progestogen (Etonogestrel) for Male Hormonal Contraception

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The effect of a novel combination of oral etonogestrel (ENG) and im testosterone decanoate (TD) on suppression of gonadotropins and spermatogenesis as a potential lead for male contraception was investigated. Healthy male volunteers were randomized into two groups receiving 300 μ g ENG daily and 400 mg TD every 4 (n = 55) or 6 (n = 57) wk for 48 wk. At wk 48, all men except one in the 6-wk group suppressed sperm concentration to less than 1 million/ml. Faster suppression occurred in the 4-wk group. Gonadotropins were suppressed in both groups and most consistently in the 4-wk group. During treatment, trough testosterone levels increased into the normal range in the 4-wk group but remained just below nor-

mal in the 6-wk group. All peak levels were within the normal range. After treatment cessation, recovery of sperm counts and gonadotropins to normal levels occurred in both groups. Minor effects on weight and cholesterol were noted. Fourteen subjects withdrew because of an adverse event with those possibly related to the study medication reported more frequently in the 6-wk group (nine vs. one). In conclusion, the combination of 300 μ g ENG with 400 mg TD every 4 wk was superior in terms of efficacy, hormone profiles, and safety. This represents a promising approach to male hormonal contraception. (*J Clin Endocrinol Metab* 90: 2042–2049, 2005)

THE STUDY OF male hormonal contraception has been making gradual progress over the last three decades. Suppression of gonadotropins by various pharmacological means, *i.e.* androgen alone (1, 2), progestogen-androgen combinations (3–6), and GnRH antagonist in combination with testosterone enanthate (7), has been shown to be both effective and reversible in suppressing spermatogenesis in healthy young men.

With most regimens using androgens alone, suppression to azoospermia occurred in 40–70% of Caucasian men, although higher efficacy has been achieved in Chinese subjects (8). The nonuniform suppression of spermatogenesis with androgen alone suggested this approach may not be optimal for Caucasian men.

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Abbreviations: Bio-T, Bioavailable testosterone; CI, confidence interval; DHT, 5 α -dihydrotestosterone; DSG, desogestrel; E2, estradiol; ENG, etonogestrel; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LNG, levonorgestrel; M, million; MPA, medroxyprogesterone acetate; PSA, prostate-specific antigen; T, testosterone; TC, total cholesterol; TD, T decanoate; TE, T enanthate.

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Progestogen-androgen combinations have been studied with better results. Many progestogens have now been combined with androgen, including medroxyprogesterone acetate (MPA) (6), levonorgestrel (LNG) (4, 9), cyproterone acetate (5, 10), desogestrel (DSG) (11–13), and norethisterone enanthate or acetate (3, 14, 15). The additive/synergistic effect between progestogens and androgens in these combinations is important both in increasing effectiveness and allowing lower androgen doses to be used. A limitation of progestogen-androgen trials to date has been the small numbers of men studied.

Etonogestrel (ENG) is the product of DSG metabolism after oral administration via 3 α -hydroxy and 3 β -hydroxy-desogestrel in liver microsomes (16). DSG is a potent and highly selective synthetic progestogen (17). Both DSG and ENG have been used safely and effectively in female contraceptives (*e.g.* Marvelon and Implanon) for some years. The application of DSG/ testosterone (T) combinations in men as hormonal contraception has been studied by three groups (11–13). A 300- μ g oral dose of DSG was found to be most effective in sperm suppression without significant side effects. A pharmacokinetic study in females found the optimal oral dose of ENG to be equal to DSG (18). Experience with

ENG in men has been in the study of ENG rods in combination with androgen (19, 20).

Testosterone decanoate (TD) is the major component of Sustanon 250, which has been used safely in the treatment of male hypogonadism for over 40 yr. A preliminary dose-finding study in eight hypogonadal men using 400 mg TD found that the T concentration was restored into the physiological range for 4–6 wk (NV Organon, unpublished). An additional study in healthy men involving repeated administration confirmed that this was an appropriate dosage and interval (20). This prolonged duration of action compared with T enanthate (TE, weekly injection required) allows for less frequent injections. Supraphysiological T excursions that are well documented with TE may be minimized, therefore reducing potentially adverse androgenic effects.

The study hypothesis is that 300 μ g oral ENG daily in combination with injectable 400 mg TD every 4 or 6 wk will be effective in suppression of gonadotropins and sperm concentration to less than 1 million/ml (M/ml). The aims of the study are to investigate this androgen-progestogen combination as a potential future male hormonal contraceptive and make a preliminary assessment of safety in men.

Subjects and Methods

Subjects

One hundred twelve healthy male volunteers were recruited from six centers (19 Helsinki, 13 Turku, 22 Münster, 20 Edinburgh, 20 Manchester, and 18 Brussels). The inclusion criteria included age at least 18 yr and no more than 45 yr; normal mental and physical health; body mass index (BMI) from 18–30 kg/m²; normal semen analysis on two occasions at least 2 wk apart [examination within 60 min, based on World Health Organization (WHO) criteria for sperm density and WHO criteria or local reference ranges for sperm motility and morphology (21)]; normal hormone (FSH, LH, and T) levels based on local reference ranges; and willingness to provide written informed consent. Men in a heterosexual relationship at study inclusion had to be willing to use a reliable form of contraception. The exclusion criteria included use of investigational drugs within six months before screening and any use of lipid-lowering drugs or prolonged use of hepatic microsomal enzyme-inducing anticonvulsant medication or other drugs known to interfere with the pharmacokinetics of steroids.

Study design

This was a phase IIb, open-label, randomized multicenter trial. The six participating centers obtained approval from the relevant local research ethics committees. After initial screening, subjects were randomized into two treatment groups receiving 1) 300 μ g oral ENG daily and 400 mg TD every 4 wk (4-wk group) or 2) 300 μ g oral ENG daily and 400 mg TD every 6 wk (6-wk group). Subjects were randomized centrally into the two treatment groups via an interactive voice response system. This was based on a minimization method that should result in a difference of fewer than three subjects between the two treatment groups. No stratification was applied.

Treatment phase duration was 48 wk and was followed by a 24-wk recovery phase. The primary endpoint was suppression of sperm to less than 1 M/ml, and the secondary endpoint was gonadotropin suppression. Physical examination and monitoring of routine hematological/biochemical analyses were made at wk 8, 24, 36, and 48 during the treatment phase and wk 4 and 24 of the recovery phase in both groups.

All subjects provided written informed consent at entry to the study. The local institutional review boards reviewed and approved the study protocol and consent forms at each center. The subjects were required to use reliable, additional contraception at study commencement.

Medications

The daily dose of ENG for all subjects was 300 μ g, administered as two 150- μ g tablets. NV Organon supplied both ENG (150- μ g) tablets and TD (400 mg in 2 ml of castor oil). Diary cards were supplied to be completed daily by the subject. The investigator cross-checked and collected the diary cards and blister packs at each visit to evaluate compliance during the study. Each visit corresponded with a blood test and/or semen analysis and/or TD injection. Compliance was calculated as the actual number of tablets taken or injections received, divided by the total number of tablets or injections scheduled. In subjects who discontinued the trial, the total number of tablets and injections scheduled would be calculated to the day before the discontinuation date. TD was administered as a single deep im injection every 4 or 6 wk.

Clinical monitoring

Subjects provided regular blood and semen samples, and any adverse events or concomitant medications were recorded. Physical examination included general assessment, weight, blood pressure, testicular size, and prostate assessment. Testicular size was assessed by orchidometer. Prostate assessment was by digital rectal examination and, where possible, transrectal ultrasonography to assess total prostate volume. Both were performed after blood sampling for prostate-specific antigen (PSA). Sexual function was assessed by the Derogatis questionnaire (22). Questions concerning tolerability of the TD injections were completed. The questions were in relation to any swelling, redness, induration, or tenderness occurring after the injection. All these assessments were made at screening and treatment wk 8, 24, 36, and 48 and recovery wk 4 and 24. A brief satisfaction questionnaire was completed at the end of the recovery phase.

Semen analysis

Semen collection and analysis of semen volume, sperm density, motility, and morphology were carried out according to the WHO Laboratory Manual for the Examination of Human Semen Sperm-Cervical Mucus Interaction (21). Where reference ranges for motility and morphology had been established locally in a fertile male population, these were used if different from WHO criteria. Two normal semen samples were required during the screening period. Throughout the treatment and recovery phases, semen samples were provided every 4–6 wk. Azoospermia was verified by centrifugation (15 min at 3000 \times g) of the entire semen sample. The resulting pellet was then thoroughly examined to exclude the presence of any sperm. Subjects completed follow-up when at least one sperm concentration had reached more than 20 M/ml.

Blood tests

During the screening phase, routine hematology (hemoglobin, hematocrit, white cell count, and platelets), biochemistry [renal and liver function; albumin; total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C); triglycerides; glucose; and glycosylated hemoglobin], PSA, and hormone analyses were carried out. The routine hematological, biochemical, and PSA measurements were arranged locally by each participating center throughout the duration of the trial. Hormone assays were undertaken at a central laboratory and included FSH, LH, total and bioavailable T (T and bio-T), 5 α -dihydrotestosterone (DHT), estradiol (E2), and SHBG. During the treatment phase, samples for hormone assay were taken at wk 1, 2, 4, 8, 16, 17, 18, 20, 24, 36, and 48 in the 4-wk group (trough samples at wk 4, 8, 16, 20, 24, 36, and 48 and peak samples at wk 1, 2, 17, and 18) and wk 1, 2, 4, 6, 8, 12, 18, 19, 20, 24, 36, and 48 in the 6-wk group (trough samples at wk 6, 12, 18, 24, 36, and 48 and peak samples at wk 1, 2, 19, and 20). Measurement of ENG was made at wk 4, 8, 24, 36, and 48 and 4, 6, 24, 36, and 48 in the respective groups. Blood sampling was to occur before ingestion of ENG or administration of injectable TD where possible. Fasting samples were taken when the lipid profiles and glucose were measured at screening and wk 8, 24, 36, and 48 of the treatment phase.

During the recovery phase, fasting samples were taken for all parameters except ENG at wk 4 and 24 in both groups.

Hormone assays

All serum samples were stored at -20 C at the individual centers. Samples were shipped on dry ice to a central laboratory of Organon for assay. Serum gonadotropins, E2, and SHBG were determined by the Delfia fluoroimmunoassay (Delfia, PerkinElmer Wallac, Turku, Finland). Assay sensitivities were 0.25 U/liter, 0.52 U/liter, and 49.93 pmol/liter, and intra- and interassay coefficients of variation were 1.9–7.6, 2.6–4.9, and 17.3–34.1% for FSH, LH, and E2, respectively. The lower limit of quantification for SHBG was 6.25 nmol/liter with intra- and interassay coefficients of variation of 3.2–5.0%. T and DHT were determined by capillary gas chromatography-mass spectrophotometry. The assay sensitivities were 0.35 nmol/liter for T and 0.34 nmol/liter for DHT with intra- and interassay coefficients of variation of 4.5–21.9 and 8.3–11.7%, respectively. Bio-T was determined after precipitation of the SHBG-bound T fraction by ammonium sulfate, and therefore no lower limit of detectability is available. The intra- and interassay coefficients of variation for bio-T was 2.3–7.7%. ENG was measured by in-house RIA (NV Organon), and the lower limit of quantification was 30.0 pg/ml.

Statistical analysis

NV Organon performed all statistical analyses using the statistical package SAS version 8.2. The intention-to-treat analysis is presented. Frequencies of subjects with suppression of sperm concentration to a specified level and a certain time point were compared by means of a Fisher exact test. A cube root scale for sperm concentration is used (Fig. 1) to allow greater clarification of low concentrations. Survival analyses were performed, and because of departure from the proportional hazards assumption, Wilcoxon tests were used for comparison. Mean values of sperm densities, hormones, biochemistry, hematology, and physical parameters were analyzed by repeated-measures ANOVA and paired *t* tests. *P* values presented for hormones, biochemistry, hematology, and physical parameters were not corrected for multiple testing; however, multiplicity was taken into account by regarding a result statistically significant if *P* < 0.0001 (which would correspond to Bonferroni corrected *P* values < 0.05). Values were expressed as the arithmetical mean ± SEM. Hormone values below the detection level were allocated the value of half of the lower limit of detection.

Results

Subjects

Among the 112 men recruited into the study, 55 were randomized to the 4-wk group and 57 to the 6-wk group. The age (mean ± SEM) of subjects was 30.2 ± 0.85 and 31.8 ± 0.82 yr in the 4- and 6-wk groups, respectively. In total, 99 (88%) men completed 24 wk of treatment, and 89 (79%) men com-

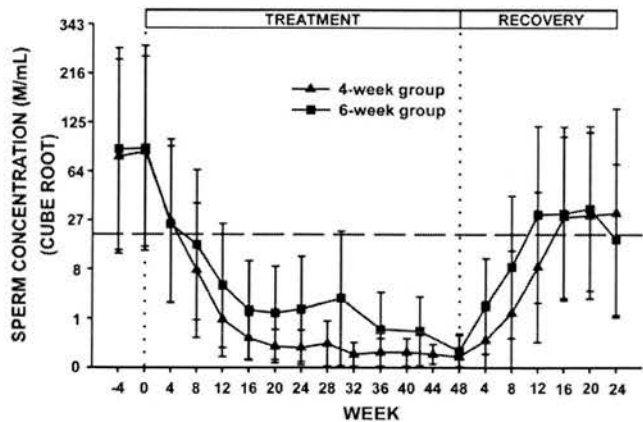


FIG. 1. Sperm density (cube root scale) during the screening (wk -4 and 0), treatment (wk 4–48), and recovery (wk 4–24) phases in two groups of healthy men receiving 300 µg oral ENG daily in combination with 400 mg TD every 4 (▲) or 6 (■) wk. The reference line represents normal sperm density of 20 M/ml. Values are the mean ± SEM.

pleted 48 wk of treatment (46 and 43 in the respective groups).

Compliance

Subjects in both groups were highly compliant with treatment. The percentage compliance with tablet ingestion and injection was 98–100% in both groups.

Sperm concentration

Mean sperm densities suppressed rapidly into the oligozoospermic range in both groups (Fig. 1 and Table 1). The extent and rate of fall in sperm density were greater in the group receiving 300 µg of ENG with 4 weekly injections of TD. Thus, at treatment wk 8, 62.3% (33 of 53) of men in the 4-wk group were suppressed to less than 1 M/ml compared with 33.3% (18 of 54) in the 6-wk group (*P* = 0.004). At treatment wk 16, sperm concentrations in all except three men in the 4-wk group (94.3%, 50 of 53) were suppressed to less than 1 M/ml compared with 83.0% (39 of 47) in the 6-wk group (*P* = 0.108). The percentage of men responding continued to increase to 98.0% (49 of 50) and 87.5% (42 of 48) at wk 24 (*P* = 0.057), and at the end of treatment (48 wk), all men were less than 1 M/ml, except one in the 6-wk group who had a sperm concentration of 1.3 M/ml. The pattern of suppression to azoospermia followed a very similar pattern (Table 1). Seventy percent (35 of 50) and 54.2% (26 of 48) of subjects at treatment wk 24, and 95.3% (41 of 43) and 82.5% (33 of 40) at wk 48 achieved azoospermia in the 4- and 6-wk groups, respectively.

The faster suppression in the 4-wk compared with the 6-wk group was also reflected in the Kaplan Meier curves (*P* = 0.019; Fig. 2). The median time to attain the sperm density suppression target of less than 1 M/ml was approximately 8 wk in the 4-wk and 12 wk in the 6-wk group.

Three subjects only demonstrated sperm concentration rebound to more than 1 M/ml after wk 24 of treatment. Two subjects saw minor increases to 1.5 M/ml (wk 42, returned to <1 M/ml again at wk 48) and 1.3 M/ml (wk 48). One subject who had achieved less than 1 M/ml at wk 20 subsequently rebounded to 2.1 and 4.6 M/ml at wk 24 and 28. The likely cause for rebound in this case was noncompliance. Six consecutive doses (300 µg daily) had been missed between wk 20 and 23 of the treatment period. Suppression to less than 1 M/ml was again achieved by wk 32.

After cessation of treatment, sperm densities recovered to normal levels in all men (Figs. 1 and 3). The median time to

TABLE 1. Percentage of subjects suppressed to several sperm density targets who received oral ENG 300 µg daily in combination with im TD 400 mg at 4- or 6-weekly intervals

Treatment group	Duration of treatment	n	% of subjects reaching the suppression targets		
			0 M/ml	<1 M/ml	<3 M/ml
4-wk	wk 8	53	18.9	62.3	75.5
	wk 16	53	50.9	94.3	98.1
	wk 24	50	70.0	98.0	100.0
	wk 48	43	95.3	100.0	100.0
6-wk	wk 8	54	9.3	33.3	44.4
	wk 16	47	44.7	83.0	91.5
	wk 24	48	54.2	87.5	93.8
	wk 48	40	82.5	97.5	100.0

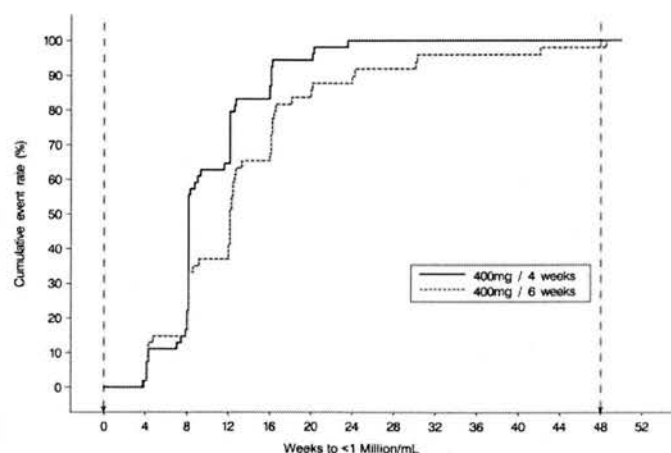


FIG. 2. Achievement of sperm density to less than 1 M/ml during the treatment phase. This is a cumulative event rate assessed between wk 0 and 48 by Kaplan-Meier estimation. The two treatment groups received 300 μ g oral ENG daily in combination with 400 mg TD every 4 (solid line) or 6 (dashed line) wk. The dashed-arrow-ended lines identify the start (wk 0) and end (wk 48) of treatment.

recovery (>20 M/ml) was 16 wk in both groups. Overall, a faster rate of recovery was evident in the 6-wk compared with the 4-wk group ($P = 0.009$; Fig. 3). Eighty-six men recovered normal sperm density within 24 wk and 22 men beyond 24 wk after the end of treatment.

LH and FSH

Both dose regimens were highly effective in suppressing circulating LH and FSH into the hypogonadotropic range (<1 IU/liter; Fig. 4). In the 4-wk group, mean LH and FSH levels declined to the lower assay detection limit after 4–8 wk of treatment; this was maintained until the end of the treatment period. In the 6-wk group, mean LH levels were less consistently suppressed during the first 24 wk of treatment but subsequently reached the lower detection limit in the

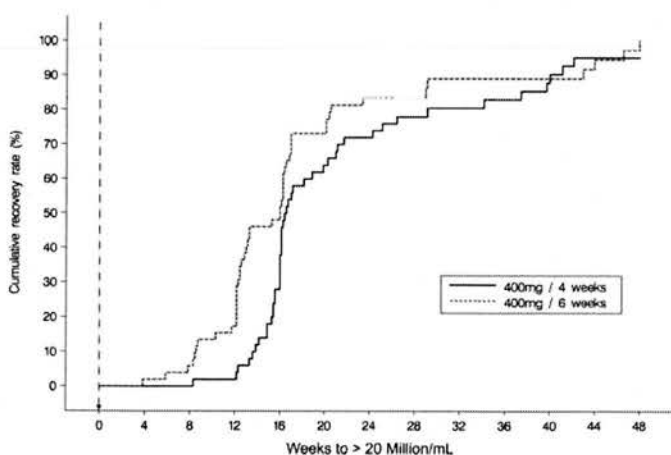


FIG. 3. Achievement of sperm density to greater than 20 M/ml after the end of treatment. This is a cumulative event rate assessed after treatment cessation (recovery phase) by Kaplan-Meier estimation. The two treatment groups had received 300 μ g oral ENG daily in combination with 400 mg TD every 4 (solid line) or 6 (dashed line) wk. The dashed-arrow-ended line at wk 0 identifies the end of the treatment phase and start of the recovery phase.

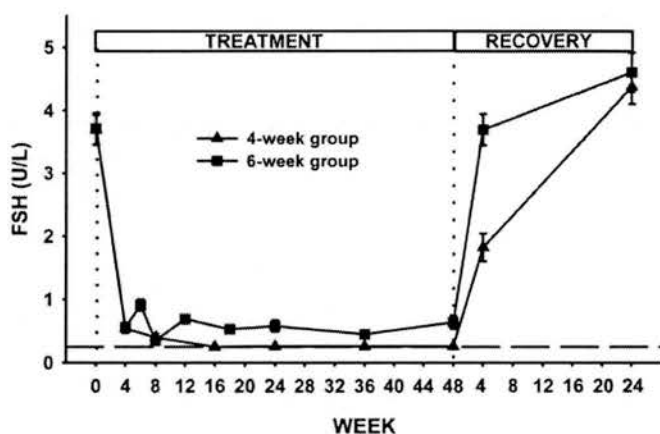
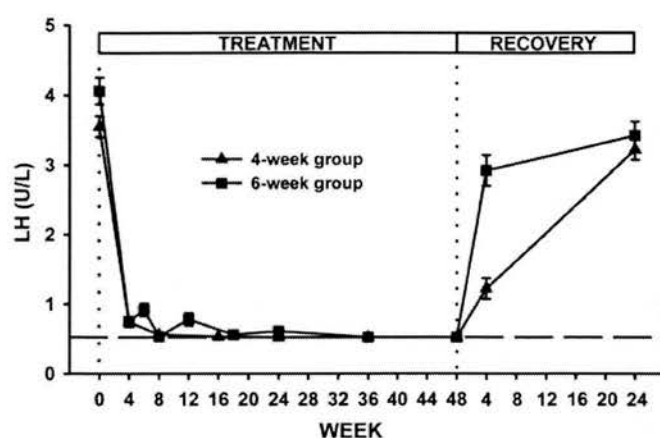


FIG. 4. Serum LH and FSH concentrations during the screening (wk 0), treatment (wk 4–48), and recovery (wk 4 and 24) phases. The two treatment groups received 300 μ g oral ENG daily in combination with 400 mg TD every 4 or 6 wk. The reference lines for LH and FSH represent the lower assay limits: LH, 0.52 U/liter; FSH, 0.25 U/liter. Values are the mean \pm SEM.

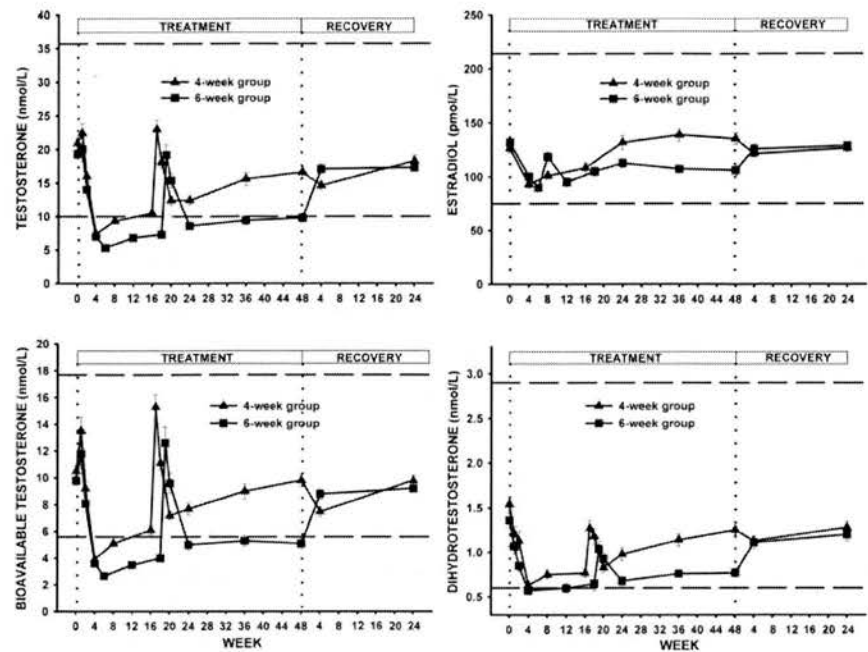
second 24 wk of treatment. FSH was not totally suppressed in all men of the 6-wk group with mean levels remaining slightly above the lower limit of assay detection (Fig. 4).

After cessation of treatment, there was rapid recovery of LH and FSH in both groups. The 6-wk group showed a faster rate of recovery as indicated by LH levels (IU/liter, mean \pm SEM) at wk 4 of the recovery phase: 1.24 ± 0.15 and 2.92 ± 0.23 in the 4- and 6-wk groups, respectively ($P < 0.0001$). This was also the case for FSH: 1.82 ± 0.22 and 3.69 ± 0.25 in the 4- and 6-wk groups, respectively ($P < 0.0001$). At wk 24 of recovery, LH and FSH were back to baseline levels in both treatment groups.

Other hormones

Mean T concentrations declined to below the normal range in both treatment groups at 4 or 6 wk after the first TD injection (Fig. 5). The trough T levels (nmol/liter, mean \pm SEM), immediately before the second TD injection, were 7.4 ± 0.38 in the 4-wk group and 5.3 ± 0.32 in the 6-wk group. Subsequently, trough T concentrations gradually increased in both groups with the 4-wk group rising to the normal range after the third injection (wk 8), but trough T levels in

FIG. 5. Total T, bio-T, DHT, and E2 concentrations during the screening (wk 0), treatment (wk 4–48), and recovery (wk 4 and 24) phases. The two treatment groups received 300 µg oral ENG daily in combination with 400 mg TD every 4 or 6 wk. The reference lines represent normal ranges: total T, 10–35.7 nmol/liter; bio-T, 5.6–17.7 nmol/liter; DHT, 0.6–2.9 nmol/liter; E2, 74.9–214.1 pmol/liter. Values are the mean ± SEM. The values represent pre-TD injection sampling at all points except wk 1 and 2 (both groups), wk 17 and 18, and wk 19 and 20 in the 4- and 6-wk groups, respectively.



the 6-wk group remained just below the normal range for the entire treatment period. Peak T concentrations were assessed after the first and fifth (4-wk group) or fourth (6-wk group) TD injection and were within the normal range in both treatment groups. SHBG decreased by approximately 35–40% in both treatment groups (Table 2). Bio-T, DHT, and E2 showed a comparable pattern to total T (Fig. 5). All hormones re-

turned to the normal range (group means and individual values) after cessation of treatment.

ENG

ENG concentrations were highly variable throughout the treatment phase. Mean ENG concentrations (pg/ml, mean ±

TABLE 2. Clinical and biochemical parameters at wk 0 and 48 of the treatment phase and at the last assessment of the recovery phase

	4-wk group			6-wk group		
	Treatment		Recovery	Treatment		Recovery
	wk 0	wk 48	Last assessment	wk 0	wk 48	Last assessment
n	55	45	53	57	41	51
Left testicular volume (ml)	23.56 (0.78)	16.45 (0.70) ^a	22.12 (0.71)	23.99 (0.80)	16.56 (0.88) ^a	23.38 (0.84)
Right testicular volume (ml)	23.86 (0.86)	17.02 (0.81) ^a	22.81 (0.81)	24.31 (0.74)	16.91 (0.85) ^a	23.77 (0.87)
Prostate volume (ml)	17.58 (0.86)	18.14 (1.04)	19.07 (1.04)	18.59 (0.67)	18.63 (1.00)	18.76 (0.77)
PSA (µg/liter)	0.79 (0.05)	0.84 (0.05)	0.87 (0.10)	0.73 (0.05)	0.81 (0.09)	0.78 (0.07)
Weight (kg)	78.40 (1.26)	82.73 (1.72)	80.42 (1.62)	79.88 (1.36)	81.95 (1.72)	80.86 (1.52)
SHBG (nmol/liter)	37.2 (1.94)	24.4 (1.69) ^a	35.2 (2.08)	36.1 (1.85)	25.7 (1.43) ^a	33.7 (1.91)
Hb (g/liter)	152.1 (0.85)	157.8 (1.39)	152.6 (1.13)	150.5 (2.29)	153.5 (1.30)	150.6 (1.08)
Hematocrit	0.45 (0.003)	0.46 (0.004)	0.44 (0.003)	0.45 (0.004)	0.45 (0.004)	0.45 (0.004)
TC (mmol/liter)	5.07 (0.14)	4.64 (0.14)	5.03 (0.15)	5.04 (0.11)	4.66 (0.12)	4.96 (0.13)
HDL-C (mmol/liter)	1.41 (0.04)	1.10 (0.03)	1.35 (0.03) ^a	1.43 (0.04)	1.22 (0.06)	1.36 (0.04)
LDL-C (mmol/liter)	3.15 (0.13)	3.84 (0.85)	3.25 (0.23)	3.08 (0.12)	2.89 (0.13)	3.07 (0.12)
Triglyceride (mmol/liter)	1.10 (0.06)	1.14 (0.10)	1.35 (0.23)	1.17 (0.07)	1.20 (0.09)	1.23 (0.09)
HbA1c (%)	4.93 (0.07)	5.05 (0.07)	5.03 (0.07)	4.88 (0.05)	4.95 (0.09)	5.03 (0.06)
Glucose fasting (mmol/liter)	4.82 (0.06)	4.99 (0.10)	4.92 (0.08)	4.79 (0.08)	4.90 (0.10)	4.99 (0.08)
ALAT (U/liter)	25.5 (2.09)	27.0 (2.15)	29.5 (3.74)	27.0 (2.15)	26.4 (2.67)	30.0 (3.30)
ASAT (U/liter)	24.8 (1.50)	21.6 (0.98)	24.7 (2.14)	23.7 (1.25)	22.1 (1.56)	25.5 (1.60)
ALP (U/liter)	121.5 (9.07)	107.8 (9.05)	124.2 (9.24)	118.4 (7.18)	111.7 (8.39)	120.4 (7.80)
γ-GT (U/liter)	20.7 (1.44)	23.5 (1.89)	25.8 (3.19)	28.5 (2.49)	35.0 (4.14)	30.34 (2.84)
Bilirubin (µmol/liter)	12.98 (0.87)	15.26 (1.07)	14.08 (0.93)	12.91 (0.67)	14.99 (1.26)	12.20 (0.91)
LDH (U/liter)	331.4 (14.0)	335.4 (14.8)	320.4 (14.5)	331.8 (14.0)	327.8 (20.3)	323.0 (15.2)

All values shown represent the mean (SEM). Testicular volumes were assessed by orchidometer. Prostate volume was via ultrasonography and was not measured at all centres (n = 23–31). Last assessment could be at wk 16, 20, and 24 of the recovery phase. Hb, Hemoglobin; HbA1c, glycosylated hemoglobin; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; ALP, alkaline phosphatase; γ-GT, γ-glutamyl transferase; LDH, lactate dehydrogenase.

^a Significant difference to baseline value with *P* < 0.0001. There were no significant differences (*P* < 0.0001) between the treatment groups in any parameter.

SEM) at wk 24 were 1329 ± 203.7 and 1590 ± 164.6 and at wk 48 were 676 ± 176.0 and 1071 ± 254.1 in the 4- and 6-wk group, respectively. It is likely that many samples were not taken before tablet ingestion as instructed, and consequently these were not trough levels. Mean trough levels could be estimated from these data and were approximately 500 pg/ml.

Physical

Testicular volumes, measured clinically by orchidometry, decreased to a comparable extent ($\sim 30\%$) in both treatment groups and recovered to baseline values after cessation of treatment (Table 2).

Prostate volumes were measured in 29 and 31 men in the 4- and 6-wk groups, respectively, with variation during treatment around the baseline values without any significant change in either group (Table 2).

Weight gain was observed in both treatment groups but was more pronounced in the 4-wk group with a mean increase body weight of 5% compared with 2% in the 6-wk group at the end of the treatment period (see also Table 2). At the last assessment of the recovery phase, the mean weight had returned to near baseline values.

Over 48 wk of treatment, acne or worsening of acne was reported in 16 (29.1%) and eight (14.0%) subjects in the 4- and 6-wk group, respectively. One subject in the 6-wk group reported gynecomastia.

Hematology, biochemistry, and lipids

The laboratory results for the two treatment groups are presented in Table 2. Mean hemoglobin increased in the 4-wk group from 152.1 g/liter at baseline to 157.8 g/liter at wk 48 ($P = 0.0005$) and from 150.5 to 153.5 g/liter between baseline and wk 48 in the 6-wk group ($P = 0.05$). No significant changes were seen in mean hematocrit values. TC and HDL-C decreased by 10% ($P < 0.0001$) in both groups and 22 and 17% ($P < 0.0001$) in the 4- and 6-wk groups, respectively. These values had returned to baseline levels at the end of the recovery phase (Table 2). No significant change occurred in either LDL-C or triglycerides. A reduction in alkaline phosphatase within the normal range was observed in both groups. There were no clinically significant changes in liver transaminases, bilirubin, PSA, or glycosylated hemoglobin (Table 2).

Discontinuations and side effects

A total of 23 subjects (21%) withdrew from the study, nine in the 4-wk group and 14 in the 6-wk group. The reasons for withdrawal were occurrence of an adverse event (14 subjects), personal (eight subjects), and failure to achieve azoospermia at wk 24 (one subject). More men withdrew from the study because an adverse event in the 6-wk compared with the 4-wk group: 10 vs. four. Moreover, adverse events in the 6-wk group were more likely to be related to the study medication, e.g. depression (3), emotional lability (2), nervousness (1), gynecomastia (1), increased sweating (1), and decreased libido (1), whereas in the 4-wk group, one subject discontinued because of nervousness. Other adverse

events that were considered unlikely to be related to the trial medication but led to discontinuation were atrial fibrillation (1), constipation (1), umbilical hernia (1) (all in the 4-wk group) and accidental injury (1) (in the 6-wk group).

Among possible side effects that did not lead to discontinuation, acne, increased sweating, mood changes, and libido changes were most frequently reported.

Questionnaires

The Derogatis questionnaire on sexual function did not show any changes in mean subscores and total scores throughout treatment, nor were there any differences between the treatment groups (data not shown).

At the last assessment, all subjects (including dropouts) were asked whether they were satisfied with the treatment, and 77% in the 4-wk and 60% in the 6-wk group answered yes. Similarly, 85 and 72% would recommend it to others, and 77 and 50% in the two respective treatment groups would definitely or probably use it if it were on the market. The injections were well tolerated.

Discussion

This is the first study in men using oral ENG in combination with injectable TD. Oral ENG 300 μ g daily in combination with 400 mg TD every 4 wk results in efficient, safe, and reversible spermatogenesis suppression. Unlike most previous progestogen-androgen combination studies we have included group sizes exceeding 15 men per treatment group and continued treatment beyond 24 wk. In the 4-wk group, severe oligozoospermia of less than 1 M/ml (the primary end point) was achieved in 98 and 100% and azoospermia in 70 and 95.3% after 24 and 48 wk of treatment, respectively. These results are comparable with previous smaller studies of oral DSG and T combinations with 67–100% of subjects achieving the target of less than 1 M/ml and 57–100% azoospermia (11–13). These results are also comparable to other progestin-testosterone combinations, e.g. depot MPA plus T implant (23) and norethisterone enanthate plus T undecanoate (14, 15). Furthermore, the present results support that spermatogenesis suppression can be maintained in the majority of subjects for up to 48 wk without escape above 1 M/ml by this oral/injectable combination. The degree and maintenance of sperm suppression attests to the overall high level of compliance with this regimen.

Previous studies have demonstrated that suppression to severe oligozoospermia (<1 M/ml) provides high contraceptive efficacy (1, 2, 8, 23). The WHO studies on 670 men showed that contraceptive failure rate was proportional to residual sperm output (1, 2). The reported pregnancy rates were 0.8 per 100 person-years [95% confidence interval (CI), 0.02–4.5] for azoospermia and 8.1 per 100 person-years (95% CI, 2.2–20.7) for oligozoospermia (<3 M/ml). A combined pregnancy rate of 1.4 per 100 person-years (95% CI, 0.4–3.7) for sperm density 0–3 M/ml is comparable to the failure rate for female oral contraceptives. In a recent efficacy study in Chinese men using testosterone undecanoate alone (8), no pregnancies resulted during the efficacy phase of the study in men suppressed to 3 M/ml. Sperm rebound occurred in six men, and one pregnancy occurred. These studies pro-

vided the evidence for the consensus that less than 1 M/ml is an appropriate target for suppression of spermatogenesis (24). The first efficacy study using a progestogen-androgen regimen recently reported no pregnancies in 426 person-months of treatment (23) using less than 1 M/ml as entry criterion for the efficacy phase. The degree of spermatogenic suppression found in the present study is therefore likely to confer a similar degree of contraceptive efficacy.

There was a clear difference in the rate of suppression between the groups (Figs. 1 and 2), highlighting the importance of the androgen component of any progestogen-androgen combination. This is because of the less consistent suppression of LH and FSH in the 6-wk compared with the 4-wk group. LH and FSH were above the assay lower detection limit in all subjects who failed to suppress to less than 3 M/ml at wk 24. The degree of gonadotropin suppression has been shown to relate to residual sperm production in several previous studies of male hormonal contraception (25). Therefore, in the first 24 wk of treatment the lower percentage of men reaching the sperm suppression targets in the 6-wk group was most likely a consequence of inadequate gonadotropin suppression (Fig. 4).

With continued treatment, the difference in percentage responding between the groups narrowed, such that by wk 48 there was no longer any difference in achieving the target of less than 1 M/ml. The continued suppression beyond 24 wk is an important new finding because most previous studies did not extend treatment after this time. Although 400 mg TD every 6 wk was clearly suboptimal, the progressive suppression occurring after 24 wk could potentially be explained by the increasing trough T levels toward the normal range. This would support the necessity for an adequate androgen dose in any progestogen-androgen regimen. Another possible explanation for the continued spermatogenic suppression could be a gonadotropin-independent action of progestin on the testis.

The median time to recovery of 16 wk in the present study is comparable to previous studies (range 16–20 wk) (1, 8, 23). Recovery was faster in the 6-wk compared with the 4-wk group. The difference in the rate of spermatogenesis recovery was reflected in the rise in gonadotropin after treatment cessation. The faster recovery rate seen in the 6-wk group may indicate that the rate of recovery is related to the duration and degree of spermatogenesis suppression during treatment. Furthermore, the shorter injection interval in the 4-wk group (last injection wk 44 *vs.* wk 42 in the 4- and 6-wk groups) would potentially result in a continued suppressive effect from exogenous T in the initial weeks of the recovery phase (starting at wk 48). In Fig. 1, mean sperm densities at the end of the recovery phase appear lower than at baseline. At and beyond wk 16 recovery, subjects who had achieved a sperm concentration of at least 20 M/ml completed the trial. Because they did not provide any additional semen samples, this results in artificially low mean sperm densities after wk 16 because all recovered men with normal sperm concentration are no longer represented.

In this study, LH and FSH recovered to the normal range by wk 24 in the recovery phase in all subjects. However, in 20% of men, recovery of spermatogenesis occurred after wk 24 recovery phase. In this group, all except three subjects

were recovered before or by wk 52 recovery. The final three subjects subsequently recovered at wk 56, 69 (no assessment after wk 46 until then), and 124 (subject had relatively low baseline counts). These findings of apparent late recovery may not be different from previous studies because there is often incomplete data collection with increased duration of the recovery phase, and those slow to recover are more likely to be lost to follow-up. Although it is not possible to say that slow recovery is specific to the present treatment or to any other hormonal treatment, the possibility of direct action on the testis needs to be considered because gonadotropins had normalized. Plasma membrane progesterone receptors are detectable in the testis (26, 27), and various progestins can compete for androgen receptor binding with T (28), down-regulate Leydig cell LH receptors (29), and inhibit Leydig cell steroidogenic enzyme (30) and 5 α -reductase activity (31). More research on progestins in men is needed.

The ENG-TD regimen resulted in decreases in both TC and HDL-C. The changes observed were similar to those reported in previous studies of DSG-TE (11, 12). TC and HDL-C decreased by 10 and 17–22%, respectively, compared with the 9–11 and 20–23% reported in the DSG studies (11–13). There was no difference in any lipid parameter between the groups. The changes in TC and HDL-C were apparent at wk 8 (data not shown) and returned to baseline during the recovery phase. T alone has been reported to lower HDL-C in healthy men receiving 200 mg TE by 13–18% with no observed change in TC, LDL-C, or triglycerides (2, 32, 33). The effect of different progestogen/androgen combinations in male hormonal contraception has been variable because of the compounds and doses used. Although studies of DSG and LNG have consistently found lowering of both TC and HDL-C (9, 11, 12), the recent efficacy study using depot MPA (23) found no significant lipid changes. The clinical significance of TC and HDL-C changes (within the normal range) in response to exogenous sex steroids in terms of cardiovascular risk is unknown (34).

Weight gain proportional to increasing DSG dose (150 or 300 μ g DSG plus 100 mg TE) has previously been demonstrated (11, 12). Here, we found a small difference in weight gain between the 4- and 6-wk TD groups (+5 and +2% in the 4- and 6-wk group, respectively; not significant). However, whether the weight gain represents lean or fat mass increase or both is currently unclear. LNG alone had no effect on lean mass and increased abdominal fat mass, but LNG and TE increased total and regional lean body mass but had no effect on abdominal fat mass (35). The effect of LNG upon fat mass appeared to be opposite to that of T. Further study of body composition in hormonal male contraception is currently in progress.

Mood-related events leading to discontinuation appeared to be more prevalent in the 6-wk group. This may indicate that 400 mg TD at 6-weekly intervals is an inadequate dose; the trough T levels in this group corroborate this. A study currently underway is including a placebo group to allow further objective assessment of adverse events associated with an ENG-T combination.

In conclusion, the novel combination of oral ENG and im TD suppressed gonadotropin and spermatogenesis with high efficacy over a 1-yr treatment period. A dose of 300 μ g

ENG combined with 400 mg TD every 4 wk is superior to 300 µg ENG combined with 400 mg every 6 wk in terms of efficacy, hormone profiles, and safety. This represents a promising approach to male hormonal contraception.

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